AN ABSTRACT OF THE DISSERTATION OF

Tyler Barker for the degree of Doctor of Philosophy in Exercise and Sport Science presented on April 2, 2009.
Title: Oxidative Stress and Muscle Dysfunction Following Anterior Cruciate Ligament Surgery.

Abstract approved:

Maret G. Traber

Despite the advances in surgery, physical therapy, and pharmaceutical agents, muscle dysfunction (i.e., atrophy and weakness) continues to impair recovery from an anterior cruciate ligament (ACL) injury and surgery. Ischemia-reperfusion injury during surgery and the subsequent limb disuse are two events experienced by patients having ACL surgery. Oxidative stress and inflammation mediate muscle dysfunction; both of which can be modulated by antioxidants. The purpose of this project was to test the hypothesis that vitamin E and C supplementation would ameliorate muscle dysfunction following ACL surgery by attenuating the increase in mediators of muscle dysfunction.

A randomized, double-blind, placebo-controlled study was conducted in men who received one of two supplements: 1) antioxidant (AO; 400 IU of vitamin E and 1000 mg vitamin C per day), or 2) matching placebo (PL) starting ~2-weeks prior to (baseline) and concluding 3-months post-surgery. Lower limb strength and skeletal muscle fiber cross-sectional area measurements were used to assess muscle dysfunction; markers of oxidative stress and inflammation were evaluated in the circulation and muscle.

Plasma α-tocopherol (α-T) and ascorbic acid (AA) increased, while γ-T concentrations decreased significantly with AO supplementation. Following surgery, oxidative stress (F_2-isoprostanes), inflammation and muscle damage increased significantly in both

groups. Compared to the PL group, AO supplementation ameliorated the increase in an anti-inflammatory cytokine (interleukin (IL)-10) and the depression of a pro- to anti-inflammatory cytokine ratio (IL-6:IL-10) immediately following surgery. Elevated AA decreased in the AO group and inversely correlated with a neutrophil chemoattractant cytokine immediately following surgery.

In contrast to our expectations, a significant atrophy response from pre- to post-surgery was not observed in muscle biopsies, using histologic techniques. In fact, AO supplementation increased inducible nitric oxide synthase and myeloperoxidase expression in the muscle following surgery. Furthermore, and unlike the PL group, the recovery in peak isometric force of the injured limb within the AO group did not significantly increase from baseline to 3-months post-surgery.

In summary, AO supplementation protected against immuno-suppression (increase in anti-inflammatory cytokines), but was ineffective in lowering oxidative stress induced by surgery. Moreover, AO supplementation did not minimize, and potentially contributed to muscle dysfunction following ACL reconstructive surgery.

Oxidative Stress and Muscle Dysfunction Following Anterior Cruciate Ligament Surgery

by
Tyler Barker

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented April 2, 2009
Commencement June 2009

Doctor of Philosophy dissertation of Tyler Barker presented on April 2, 2009.

APPROVED:

Major Professor, representing Exercise and Sport Science

Chair of the Department of Nutrition and Exercise Sciences

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Tyler Barker, Author

ACKNOWLEDGEMENTS

The author would like to express his sincerest appreciation to Dr. Melinda Manore, Dr. Emily Ho, Dr. Mark Hoffman, Dr. Stephen Dickenson and Dr. Jeffrey Widrick for their patience, guidance and friendship during this project and during my time at Oregon State University (OSU). Thank you.

At OSU, I would like to thank Scott Leonard, Kate Lebold, The Department of Nutrition and Exercise Sciences, The Linus Pauling Institute and the Traber Lab. The completion of this project is a direct result of your assistance and friendship.

At The Orthopedic Specialty Hospital, I would like to express my gratitude to Dr. Roy Trawick and Dr. James Walker for their support, guidance and friendship throughout this project.

I would also like to thank Graham Burdett and Ronda Ingram for their support with the identification of potential subjects for this study; Penny Snow, Terri Covington, and Kristi Thunell for phlebotomy; Thomas Martins, Dr. Carl Kjeldsberg and Dr. Harry Hill at ARUP for their support with the measurement of various cytokines; the Central Laboratory staff at the Intermountain Medical Center (Intermountain Healthcare, Murray, UT, USA) for various clinical blood measures; Carlson Laboratories (Arlington Heights, IL, USA) for the generous donation of both the antioxidant and placebo supplements; James A. Walker for all of his support, and Janet Hansen at the Electron Microscopy Lab at the Intermountain Medical Center; and all of the subjects that participated in this study.

I would also like to thank funding support provided by Deseret Foundation at Intermountain Healthcare and USANA Health Sciences.

To my parents, Mark and Karen Koncar, your love, support, and encouragement throughout my graduate studies has been truly appreciated. Thank you and I love you.

I would like to thank our son, Chase Barker, for providing love, laughter and balance; and to our soon to be daughter, Ryanne Barker, for providing light and emotional drive.

To my beautiful wife Alison, thank you and I love you. Your support and passionate drive has been inspirational throughout my graduate studies. Without you, this would have never been possible.

I would especially like to thank Dr. Maret Traber, I would not be here today without you! You are extraordinary mentor, an inspirational scientist, and a beloved friend. You have provided direction, opportunity, positive encouragement, constructive criticism and life changing wisdom. You have been and will always be one of the most influential individuals in my life. I hope that all of your graduate students appreciate you and everything that you do. Thank you for everything.

CONTRIBUTION OF AUTHORS

Chapter 3: Scott W. Leonard performed the plasma vitamin E and C measurements. Roy H. Trawick provided subject availability from his clinic practice. Thomas B. Martins performed the cytokine measurements, with assistance from Tyler Barker. Harry R. Hill and Carl R. Kjeldsberg provided various lab resources. Harry R. Hill, Carl R. Kjeldsberg, Scott W. Leonard and Roy H. Trawick reviewed the manuscript. Maret G. Traber provided concept, idea, research design, data analysis, critical critique of writing and review. Tyler Barker provided concept, idea, research design, subject recruitment, project management, data collection, various measurements and analysis.

Chapter 4: Scott W. Leonard performed the plasma vitamin E and C, malondialdehyde, and F_2-isoprostane measurements. Roy H. Trawick provided subject availability from his practice. Thomas B. Martins performed the cytokine measurements, with assistance from Tyler Barker. James A. Walker provided assistance with research concept. Janet Hansen provided immunohistochemistry services. Katherine M. Lebold performed the plasma myeloperoxidase measurements. Ronda Ingram and Graham Burdett assisted with subject identification for later recruitment. Harry R. Hill and Carl R. Kjeldsberg provided lab resources. Harry R. Hill, Carl R. Kjeldsberg, Scott W. Leonard, Roy H. Trawick, Janet Hansen, Ronda Ingram, Graham Burdett and James A. Walker reviewed the manuscript. Maret G. Traber provided concept, idea, research design, data analysis, critical critique of writing and review. Tyler Barker provided concept, idea, research design, subject recruitment, project management, data collection, various measurements and analysis.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continues)

LIST OF TABLES

Oxidative Stress and Muscle Dysfunction Following Anterior Cruciate Ligament Surgery

CHAPTER 1. INTRODUCTION

Muscle dysfunction (i.e., atrophy and weakness) is an immediate and persistent impairment that follows of one of the most commonly injured and surgically repaired ligaments in the knee, the anterior cruciate ligament (ACL). Although altered neurological activation and reduced limb activity are important contributors to muscle dysfunction, muscle dysfunction itself continues despite modern advancements in orthopedic surgery, physical therapy and pharmaceutical agents.

Recent evidence indicates an increase in circulating oxidative stress during the reperfusion conditions immediately following surgery, and an elevation of pro-inflammatory cytokines in the knee joint fluid following an ACL injury and surgery. These are enlightening findings because oxidative stress and pro-inflammatory cytokines mediate muscle dysfunction. Fortunately, anti-inflammatory cytokines reduce the production of pro-inflammatory cytokines and provide protection against cytokine-induced muscle dysfunction. Similarly, antioxidants attenuate muscle dysfunction by ameliorating oxidative stress and the elevation of pro-inflammatory cytokines. Specifically, vitamins E and C are potent antioxidants that display immuno-modulating properties. It is currently unknown if the supplementation of vitamins E and C minimize muscle dysfunction following an ACL injury and surgery by attenuating circulating and local mediators of muscle dysfunction.

Therefore, the purpose of this project was to identify the influence of vitamin E and C supplementation on muscle dysfunction and mediators of muscle dysfunction following

an ACL injury and surgery. To pursue this purpose, we constructed the following specific aims and working hypotheses:

Specific Aim #1

A. To identify circulating biomarkers of oxidative stress and inflammatory cytokines during the reperfusion conditions that follow ACL surgery.
B. To identify the influence of vitamin E and C supplementation on circulating biomarkers of oxidative stress and inflammatory cytokines during the reperfusion conditions that follow ACL surgery.

Hypothesis

Our working hypothesis is that vitamin E and C supplementation ameliorates the increase of circulating biomarkers of oxidative stress and inflammatory cytokines during the reperfusion conditions immediately following ACL surgery.

Specific Aim #2

A. To identify inflammatory-derived oxidative stress in the musculature of the injured ACL limb prior to and 1-week after ACL surgery.
B. To identify the influence of vitamin E and C supplementation on inflammatory-derived oxidative stress in the musculature of the injured ACL limb prior to and 1-week after ACL surgery.

Hypothesis

Our working hypothesis is that vitamin E and C supplementation ameliorates the increase of inflammatory-derived oxidative stress in the musculature of the injured ACL limb 1-week post-surgery.

Specific Aim #3

A. To identify a pro-inflammatory cytokine and inducible nitric oxide synthase expression in the musculature of the injured ACL limb prior to and 1-week after ACL surgery.
B. To identify the influence of vitamin E and C supplementation on a inflammatory cytokine and inducible nitric oxide synthase expression in the musculature of the injured ACL limb prior to and 1-week after ACL surgery.

Hypothesis

Our working hypothesis is that vitamin E and C supplementation ameliorates the increase of a pro-inflammatory cytokine and inducible nitric oxide synthase expression in the musculature of the injured ACL limb 1-week post-ACL surgery.

Specific Aim #4

A. To identify circulating mediators (i.e., oxidative stress and inflammatory cytokines) of muscle dysfunction and the strength recovery of the injured limb following ACL surgery.

B. To identify the influence of vitamin E and C supplementation on circulating mediators of muscle dysfunction and strength recovery following an ACL injury and surgery.

Hypothesis

Our working hypothesis is that vitamin E and C supplementation will improve strength recovery following ACL surgery by ameliorating the increase in oxidative stress and inflammatory cytokines.

CHAPTER 2. LITERATURE REVIEW

The anterior cruciate ligament (ACL) is one of the most commonly injured ligaments in the knee. Approximately 200,000 ACL injuries occur every year with nearly half of those requiring reconstructive surgery. Despite advances in orthopedic surgery [264] and physical therapy [368], both morphological (i.e., muscle atrophy) and functional impairment (i.e., weakness) of the quadriceps persist for extended periods of time, sometimes even years [17]. Ischemia-reperfusion during surgery and limb immobilization disuse post-surgery are two pathological events experienced by individuals having ACL reconstructive surgery that may mediate muscle dysfunction (i.e., atrophy and weakness). Thus far, the pathophysiological mechanism(s) responsible for muscle dysfunction (i.e., atrophy and weakness) following ACL surgery are unknown.

The ischemia-reperfusion conditions elicited by the tourniquet procedure performed during ACL surgery exacerbate (i.e., < 1 yr) post-surgery muscle atrophy [19, 70, 260], necrosis [15] and weakness [70]. These injuries closely resemble the ischemia-reperfusion injuries of muscle atrophy [20, 59], necrosis [193, 311] and weakness [214, 294, 295, 392], and the limb immobilization- (or disuse) induced muscle atrophy [12, 41-43, 168, 262, 329, 357] and weakness [44, 109, 168, 390, 391] observed in experimental animal studies. Often ischemia-reperfusion injuries and limb immobilization or disuse outcomes are mediated by oxidative stress and inflammatory cytokines; both of which can be modulated by antioxidants [14, 113, 153, 163, 169, 170, 263, 310, 328]. Although there is an increase in circulating oxidative stress during reperfusion (5 to 120 minutes post-tourniquet removal) conditions associated with ACL

surgery [319, 388], and an increase in local (synovial fluid of the knee joint) cytokine concentrations following injury (prior to surgery) [56, 147, 158] and surgery (7-days) [401], it is currently unknown if oxidative stress or inflammatory cytokines, or both, contribute to muscle dysfunction following ACL surgery.

Anterior Cruciate Ligament

The ACL is a fibrous tissue composed of collagenous bundles that provide stability to the knee joint. Specifically, the ACL provides support to the knee joint by minimizing the anterior translocation of the tibia relative to the femur. The ACL is commonly injured through twisting motions of the knee joint or through impact on the outside part of the knee. Historically, ACL injuries that required reconstructive surgery used to be life-long debilitating injuries with persistent morphological deterioration (i.e., muscle atrophy) [17] and functional impairment (i.e., weakness) [21] of the quadriceps femoris muscle group. Fortunately, advances in orthopedic surgery (i.e., arthroscopic vs. open surgery) [264] and physical therapy (i.e., early weight-bearing and motion) [264, 368] have significantly improved the morphological and functional recovery from ACL surgery. However, despite these advances, persistent muscle atrophy and weakness continue to challenge the rehabilitation to ACL reconstructive surgery while the pathophysiology of muscle atrophy and weakness following ACL surgery remains unknown [17].

Pre-Operative Muscle Dysfunction

Prior to ACL reconstructive surgery, physical therapy typically consists of diminishing inflammation, swelling, pain and restoring the range-of-motion of the injured knee and improving the voluntary activation of the quadriceps of the injured limb [385].

Furthermore, in comparison to the non-injured limb, significant muscle (quadriceps) atrophy of the injured limb occurs prior to surgery [97]; although, findings have been inconsistent [148, 382]. Additionally, strength deficits of the injured limb compared to the non-injured are highly prevalent prior to surgery as well [7, 97, 123, 239, 240]. Thus, the dynamic impairment of muscle dysfunction is readily apparent prior to ACL surgery.

Oxidative Stress

Oxidative stress occurs when there is an imbalance between free radical production (i.e., reactive oxygen (ROS) and/or nitrogen (RNS) species) and antioxidant defenses [138]. A free radical is any species capable of independent existence that contains one or more unpaired electrons that are capable of eliciting damage. Thus, increased ROS and/or RNS production, or a decrease in antioxidant capacity, can result in oxidative stress. Both exogenous (i.e., pollution, radiation, pesticides, etc.) and endogenous (i.e., mitochondria, phagocytes, oxidases, nitric oxide synthases, etc.) sources are responsible for free radical production, and thus oxidative stress [72, 286].

Activated phagocytes are one source of oxidative stress. Activated phagocytes generate reactive oxidants (i.e., superoxide, ($O_2^{\bullet -}$)) which dismutate into other reactive oxidant (i.e., hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$)) or react with nitric oxide (NO•) to generate reactive nitrogen species such as peroxynitrite ($ONOO^-$) [138].

Nitric oxide (NO•) is a RNS that mediates a variety of biological processes (i.e., vasodilation, neurotransmission and cytotoxicity) [138]. Nitric oxide is synthesized by one of three nitric oxide synthase (NOS) enzymes. Both neuronal NOS (nNOS) and

endothelial NOS (eNOS) enzymes are expressed in skeletal muscle [114, 177] where they are predominantly localized to the sarcolemma and mitochondria, respectively [114, 177, 343]. Inducible NOS (iNOS) is expressed in human macrophages [138] and skeletal muscle [315], and as shown in experimental animal studies, increases with inflammatory cytokine stimulation (i.e., TNF-α, interferon (IFN)-γ, etc.) [37, 50, 171, 323]. iNOS expression has also been demonstrated to increase during reperfusion following ischemia [290] and during limb immobilization [399]. NO• production from the various forms of NOS and the high output of $O_2^{\bullet -}$ by neutrophils potentially creates other cytotoxic molecules (i.e., $ONOO^-$) that are deleterious to the environment where they are produced [33]. Therefore, iNOS may be an additional source reactive substances that induce muscle dysfunction following ACL reconstructive surgery.

Myeloperoxidase (MPO) is secreted from activated phagocytes [100]. This enzyme catalyzes the reaction of hydrogen peroxide and chloride ions to form hypochlorous acid ($HOCl^-$) [145]. Thus, MPO is a major source of oxidative stress derived from an inflammatory response.

During resting or with non-disease conditions, skeletal muscles produce small amounts of free radicals [299]. However, greater concentrations of free radicals causes adverse effects, including decreasing muscle force production [10, 71, 280].

Antioxidants scavenge free radicals and decrease muscle force (i.e., twitch and tetanus) in experimental animals that are not subjected to undue stress [83, 302]. However, during conditions of increased oxidative stress (i.e., increased muscle activity, ischemia-reperfusion, etc.) in experimental animals, muscle force production

deteriorates [24, 83, 85, 299], but can be minimized by antioxidants [238, 265, 349]. Excitation-contraction coupling [281], calcium sensitivity [9, 10, 250, 285] and cross-bridge mechanic [280] impairments are mechanisms that contribute to the decrease in muscle force in response to oxidative stress in experimental animals or isolated muscle preparations.

Similar to oxidative stress, NO• modulates muscle function and is produced at low levels during resting conditions [23, 176, 300]. For the most part, endogenous NO• [300], NO• donors [118, 146, 176, 274] and exogenous $ONOO^-$ [348] decrease the force production capacity of isolated skeletal muscles. However, the modulating influence of NO• on skeletal muscle becomes confusing when taking into consideration data suggesting that NO donors do not affect muscle force, shortening [8] or power output production [254, 255]; while some data indicates that NO• either increases [228] or decreases [118] muscle shortening velocity.

Various NOS blockers (7-nitroindazole, aminoguanidine) that presumably decrease NO• production [23], increase force production of skeletal muscle [176, 300]. However, findings on muscle function (i.e., muscle shortening and power output) have been inconsistent with regard to other NOS blockers (nitro-L-arginine or 7-nitroindazole) or a NOS inhibitor (N-nitro-L-arginine) [228, 254, 255].

Antioxidants

Antioxidants consist of 1) enzymes that catalytically remove free radicals and other reactive species (i.e., superoxide dismutases, catalase, peroxidases, etc.), 2) chelating

agents, such as proteins that minimize the availability of metals that can act as pro-oxidants (i.e., transferrins, ceruloplasmin, and metallothionen), 3) proteins that protect against biomolecule damage (i.e., small heat shock proteins), and 4) low molecular mass agents that scavenge reactive species (i.e., ascorbic acid (vitamin C) and α-tocopherol (vitamin E)) [138].

Vitamin E is a lipid soluble antioxidant that provides protection against the propagation of lipid peroxidation (membrane damage) [51, 138]. Vitamin C also recycles vitamin E by reducing the tocopheroxyl radical to tocopherol [47, 139] .Vitamin C is a major water-soluble antioxidant that scavenges a variety of reactive oxygen and nitrogen species [115, 138, 331]. Vitamin C is highly concentrated in neutrophils [202] and protects against the inflammatory cell derived-oxidative stress [138]. Importantly, vitamin C also acts as a co-factor for collagen synthesis.

Inflammatory Cytokines

Inflammatory cytokines are also mediators of muscle dysfunction [298]. These cytokines are secreted by a variety of cells, both inflammatory (i.e., CD4+, CD8+, monocytes/macrophages, neutrophils, lymphocytes, natural killer (NK) cells, etc.) and non-inflammatory (i.e., endothelial, skeletal muscle, fibroblasts, etc.). Inflammatory cytokines act on other cells both systemically and locally. Among the different cytokines, the actions of TNF-α are best understood with regard to its deleterious influence on muscle atrophy or protein loss [207, 220, 221, 284], weakness [6, 84, 143, 205] and its ability to stimulate oxidative stress [6, 171, 323]. TNF-α modulates interleukin-6 (IL-6), an inflammatory cytokine that increases muscle atrophy [135, 366]. However, the influence of IL-6 influence on muscle weakness without overt atrophy

and its ability to stimulate oxidative stress are unknown. Both TNF-α and IL-6 are expressed in human muscle under resting conditions [282], and TNF-α has been prominently linked to muscle pathophysiology and demonstrated to increase its expression in muscle during reperfusion from ischemia [117] and limb immobilization [399] in experimental animal studies.

Elevated but “physiologically acceptable” TNF-α concentrations directly induce skeletal muscle protein loss (total protein content and adult fast-type myosin heavy chain) and rapidly activate nuclear transcription factors (i.e., NF-κB) [212]. The TNF-α/NF-κB signaling pathway in skeletal muscle appears to be regulated by endogenous reactive oxygen species produced by mitochondria [206, 322, 323]. Originally, Schulze-Osthoff et al. [322, 323], and subsequently Li et al. [206], found that the activation of NF-κB by TNF-α is blocked by inhibitors of complex I in the mitochondrial respiratory chain, whereas inhibitors of complex III enhanced the activation of NF-κB by TNF-α, suggesting that the modulation of NF-κB by TNF-α is mediated by oxidative stress generated by the mitochondria. Specifically, NF-κB in vitro was inhibited by the addition of catalase which decomposes H_2O_2, while added H_2O_2 enhanced NF-κB activation [212]. Finally, under chronically elevated TNF-α conditions in mice, there is an increase in oxidative stress, iNOS and muscle wasting; all of which were reversed with vitamin E supplementation [50].

Pro-inflammatory cytokines and cytokine-derived pathways are also subject to regulation by anti-inflammatory cytokines. Interleukin (IL)-10 is an anti-inflammatory cytokine that is transcriptionally regulated by NF-κB, among others (i.e., cyclic adenosine monophosphate response element, a glucorticoid response element, etc.)

[99, 173, 252]. Pro-inflammatory cytokines (i.e., TNF-α, IL-1β, IL-6, etc.) [69, 359, 381] and oxidative stress [136, 198, 226] stimulate IL-10 production, while IL-10 reciprocally down-regulates cytokine production [39, 106, 107, 381] and oxidative stress [90, 179]. In experimental knockout animals, Kryzston et al. [192] demonstrated that IL-10 deficiency exacerbates muscle weakness and the subsequent pro-inflammatory cytokine increase (i.e., TNF-α, IL-1β, IL-6) following lipopolysaccharide stimulation. Thus, the pro-to-anti-inflammatory cytokine balance is critical for muscle function, as described by Divangahi et al. [89]. IL-10 plays an instrumental role in regulating endogenous cytokine production and oxidative stress. These factors have important implications for muscle function.

Inflammatory cytokines are instrumental in the coordination of the immune system. Adaptive immunity is mediated by cellular- and humoral-immune responses. In general, T helper 1 (Th1) cells induce cellular immunity, while Th2 cells promote humoral immune responses. Interleukin (IL) -2, IL-12 and IFN-γ influence Th1-type cells and the pathways they regulate [66, 224, 345], while IL-4, IL-5 and IL-10 are produced by Th2-type cells and influence Th2-type cell pathways [40, 68, 105, 172, 252]. Both Th1- and Th2-cells and cytokines are influenced by IL-10 [79, 351].

Understanding the immune system coordination of Th1 and Th2 type cytokines may have important implications on muscle dysfunction following an ACL injury and surgery because impaired Th1 and enhanced Th2 type immunological responses typically occur with aging [74, 244, 258, 306, 332, 393], a process characterized by the loss of muscle mass and strength (i.e., sarcopenia) [316].

Antioxidants may regulate the immune system. Importantly, IL-2 is a Th1-type and immune signaling cytokine that is also modulated by vitamins E and C [389]. IL-2 is a prototypical inflammatory cytokine that augments the proliferation of cytotoxic and suppressor T-cells [375], and facilitates the cytolytic activity of NK cells [92, 266, 365] and monocytes [166] by increasing production of other cytokines (i.e., IFN-γ, TNF-α, IL-6). Furthermore, IL-2 promotes B cell growth and differentiation in vitro [376]. Malmberg et al. [227] found in humans suffering from colorectal cancer a simultaneous elevation in plasma α-T and IL-2 following 2 wks of vitamin E supplementation (750 mg) combined with RDA levels of vitamin C and selenium. In healthy elderly subjects (≥ 60 y), an increase in IL-2 production was found following 30 d of vitamin E supplementation (800 mg dl-α-tocopheryl acetate) [244]. Vitamin E supplementation has also been found to restore the decrease in IL-2 production in old mice infected with influenza [142] or AIDS [380], enhance naïve T cells IL-2 production [2], and increase the expression of IL-2 in T cells obtained from both young and old mice [141]. Thus, the immuno-modulating capacity of vitamin E is clearly represented by their ability to regulate IL-2.

Anterior Cruciate Ligament Surgery Ischemia-Reperfusion Injury

ACL reconstructive surgery of necessity causes an ischemia-reperfusion injury. Ischemia refers to a decrease supply of oxygenated blood to a body organ or limb. Ischemia is followed by the subsequent re-introduction, or reperfusion, of oxygenated blood. Tourniquet-induced ischemia is necessary for ACL reconstructive surgery because it provides a bloodless operating field for the surgeon. Ischemia-reperfusion injury is multi-factorial in nature with severe systemic and local consequences that are

mediated by oxidative stress and inflammatory cytokines. The pathogenesis of the ischemia-reperfusion injuries of muscle atrophy, necrosis, damage and weakness have been well characterized in experimental animal studies [312]. However, similar information is not available for humans recovering from ACL surgery.

During ACL surgery a tourniquet is routinely administered to the upper thigh, directly over the thigh muscles. Unfortunately, the tourniquet procedure elicits both systemic and local responses, which potentially impair the morphological and functional short-term recovery to surgery [70]. The tourniquet procedure (tourniquet duration range of 40-186 minutes, 280-300 mm Hg tourniquet inflammation pressure) performed during ACL surgery likely is a factor in the decrease in thigh circumference observed 3 to12 weeks post-ACL reconstructive surgery, as compared with the a non-tourniquet procedure [70, 260]. However, by 1 year post-operation there appears to minimal if any difference in muscle size or strength with tourniquet use [70]. Furthermore, deterioration of muscle ultrastructure (i.e., separation of myofibrils) also increases with tourniquet-assisted ACL surgery and is highly dependent upon the ischemia (i.e., tourniquet) duration with muscle necrosis apparent after 90 minutes of ischemia [15]. Similar to local identifiers of muscle necrosis and damage, systemic biomarkers indicative of muscle damage (i.e., plasma myoglobin) are also elevated following the tourniquet procedure performed during elective knee surgery [162]. Finally, tourniquet-assisted ACL surgery reduces isometric (at 90º flexion) quadriceps strength 12 weeks post-ACL surgery, when compared with surgeries performed without an ACL tourniquet [70]. Although the tourniquet procedure is commonly performed during ACL reconstructive surgery, the immediate effects of tourniquet-induced ischemia include short-term (less than 6 months) muscle atrophy, necrosis and weakness.

Tourniquet induced ischemia (98 ± 14 minutes) during ACL surgery increases muscle metabolites (i.e., hypoxanthine) [267] that suggest an increase in oxidative stress occurs upon reperfusion [138]. This assumption is supported by increases in oxidative stress biomarkers (i.e., malondialdehyde (MDA), comet assay) 5-minutes post-ischemia (ischemia duration of ~74 minutes) following ACL surgery [319, 388]. The increase in MDA could be ameliorated by an intravenous infusion of the antioxidant, N-acetyl cysteine [319]. However, the major limitations of that particular study are 1) that the final outcome measurement was limited to a 5 minutes post-ischemia and 2) that the measurement of MDA is problematic using thiobarbituric acid (TBA) because TBA may cross-react with other substances [138]. Unfortunately, the basic scientific knowledge regarding the systemic and local consequences of ischemia-reperfusion injury in experimental animals have not been applied to humans with respect to ACL surgery. Thus, there is a gap in our knowledge that identifies the pathophysiological significance of oxidative stress on muscle atrophy, necrosis and weakness following tourniquet assisted ACL reconstructive surgery.

Ischemia-Injury

Ischemia Muscle Morphology, Structure and Function

The effects of ischemia on the depletion of energy metabolites, or adenosine triphosphate (ATP), are well documented [154, 155]. Moreover, it appears that prolonged ischemia duration has deleterious physiologic consequences. Prolonged ischemia duration increases muscle atrophy [16], muscle necrosis [276], ultrastructural

muscle damage (dilatation of the sarcoplasmic reticulum and T-tubules) [144] and delays the morphological recovery of skeletal muscle [294].

Muscle function in humans (maximal isometric quadriceps force production at 60º knee flexion angle) is significantly reduced immediately following brief (i.e., 1-5 minutes), or intermittent ischemia (5 sets of 5 minutes with a 3 minute rest, mean inflation pressure 280 mm Hg) [279]. Similarly, experimental animal studies demonstrate that muscle force production is reduced with ischemia [392], and progressively deteriorates with increasing ischemia duration (0, 15, 30, 45, 60,120, 180, and 240 minutes) [159, 367]. Taken collectively, longer ischemia durations are associated with greater atrophy and strength impairments.

Ischemia-Reperfusion Injury

Ischemia-Reperfusion Muscle Morphology, Structure and Function

While prolonged duration of ischemia elicit deleterious morphological and structural effects, it appears that the majority of systemic and local insult occurs during reperfusion. Muscle atrophy (muscle fiber size and mass) is detectable weeks after ischemia-reperfusion injury [20, 59]. Similarly, necrosis [193, 311], ultrastructural abnormalities (i.e., mitochondrial swelling, contracted bands, *Z*-line streamlining and organizational loss) [52, 392], sarcolemma separation from myofibrils [144], damaged myofibrils [174], and muscle injury (as determined by ^{99m}Tc-pyrophosphate) [344] are all present in post-ischemic reperfused muscles. Muscle necrosis can be detected as early as 30 minutes after a 2 hour bout of ischemia [175]. Moreover, muscle necrosis [16, 175, 193], morphological alterations (i.e., vimentin) [214] and ultrastructural

damage [111, 112] are more severely affected during reperfusion compared with ischemia alone. Interestingly, necrotic fibers also display more atrophy than viable fibers following reperfusion [175]. In addition to muscle necrosis and damage, there is also an increase in systemic biomarkers representative of muscle damage (i.e., creatine kinase and myoglobin) following ischemia-reperfusion injury [52, 95, 162, 311].

Several studies have demonstrated that reperfused skeletal muscles display distinct reductions in muscular force [214, 294, 295, 392], which can take several weeks to recover, if recovery occurs at all [294, 295, 392]. The impaired muscle function following reperfusion is also inversely correlated with ischemia duration [108], which would suggest that ischemia duration plays an integral part in functional recovery of skeletal muscle to ischemia-reperfusion injury.

Ischemia-Reperfusion Oxidative Stress

Ischemia alone does not appear to cause a significant increase in oxidative stress. Reportedly, during ischemia there are no changes in muscle glutathione or glutathione disulfide [94, 334], no changes in muscle protein carbonyls, MDA [132, 392] or hydroxy-conjugated dienes [216]. Furthermore, electron spin resonance techniques confirm a lack of increase in the production of free radicals in the muscle (rat tibialis anterior) during ischemia [272].

In contrast to ischemia, during reperfusion an increase in oxidative stress is well documented [94, 102, 132, 144, 217, 333, 334]. Specifically, reperfused skeletal muscles display decreased glutathione concentrations [334, 337], an increase in oxidized glutathione [132], an increase in the oxidized glutathione/reduced glutathione

ratio [94], increases in lipid peroxidation markers (e.g. MDA [132], TBARS [102], conjugated dienes [310]) and increases hydroxyl radicals [273]. Furthermore, the reperfusion oxidative stress response may be dependent on ischemia duration, with longer ischemia duration eliciting a greater reperfusion oxidative stress [54] and muscle damage [144].

One potential source of oxidative stress during reperfusion is xanthine oxidase. During ischemia there is a breakdown in energy metabolites (i.e., phosphocreatine and ATP) that accumulate as hypoxanthine [93, 154, 342]. The enzyme, xanthine dehyrogenase, converts to xanthine oxidase [336, 338]. Upon reperfusion, hypoxanthine is a substrate for xanthine oxidase which converts oxygen into $O_2^{\bullet -}$, a free radical and precursor to more deleterious radicals [131].

Xanthine oxidase depleting diets (tungsten-supplemented, molybdenum-deficient diet) decrease muscle xanthine oxidase and concurrently decrease vascular permeability without altering muscle myeloperoxidase (MPO) during reperfusion [338]. Other indirect inhibitors of xanthine oxidase and free radical scavengers (allopurinol or oxypurinol) do not ameliorate muscle necrosis, neutrophil infiltration [93] or biomarkers of lipid peroxidation in reperfused skeletal muscle [104], whereas neutrophil depletion with mechlorethamine decreased muscle MPO and ameliorated muscle necrosis [93]. The increase in xanthine oxidase activity is a source of free radical production in reperfused skeletal muscle, and appears responsible for endothelial dysfunction and increased vascular permeability during ischemia-reperfusion injury [190, 270, 342].

Ischemia-Reperfusion Myeloperoxidase

MPO is secreted from activated phagocytes [100] and catalyzes the formation of $HOCl^-$ [145]. Studies in rats, pig, or dogs demonstrate that reperfused skeletal muscles possess greater MPO activity than do control, non-ischemic muscles [57, 93, 169, 310, 313, 314, 324, 337]. This MPO increase was also accompanied with an increase in biomarkers indicative of oxidative stress (i.e., decrease in glutathione and increase in conjugated dienes) [131, 310, 337], muscle necrosis [93, 310] and impaired muscle force output (i.e., twitch and tetanus) [169, 170]. While the MPO response to ischemia-reperfusion elicits muscle dysfunction in experimental animal studies, it is currently unknown if there is an increase in either systemic or local MPO, or both, following ACL surgery in humans.

Ischemia-Reperfusion Inducible Nitric Oxide Synthase

NO• has emerged as a potential mediator of ischemia-reperfusion injury in skeletal muscle [64]. Following ischemia-reperfusion, eNOS and nNOS mRNA are down-regulated, but iNOS mRNA [289, 291] and protein [290] expression is increased in skeletal muscle and peripheral nerves [292]. Increased iNOS protein expression may be the result of increased iNOS-expressing leukocytes infiltrating skeletal muscle [37, 243] and/or an increase in inflammatory cytokines causing increased iNOS expression [386]. Reperfused skeletal muscles that display necrosis [291], inflammatory infiltration [65, 291] and muscle impairment [65, 271] also display increased iNOS expression. However, it is currently unknown if there is an increase in iNOS protein expression following ACL surgery.

The decrease in eNOS and nNOS during ischemia-reperfusion presumably reduces NO• production [289, 291]. Reduced NO• production via NOS blockade or by NO• scavengers potentiates muscle force output whereas increased NO• production [176] or NO• donors [146] inhibit force output. However, a certain level of NO• is essential for optimal muscle function [254, 255]. Based on the inverse correlation between NOS activity and muscle function [176] and the reduction in muscle function during reperfusion [214, 294, 295, 392], one would expect 'excessive' NOS activity during reperfusion. This assumption is supported by the increase in iNOS following ischemia-reperfusion injury [291] and likely accounts for a substantial increase in NO• production during reperfusion [243]. The increase in iNOS during reperfusion also increases with ischemia duration [289] and is ameliorated with iNOS inhibitors [65, 400] or iNOS gene deficiency [25, 291]. Ameliorating iNOS expression with iNOS inhibitors (dexamethasone or (N-[3-(aminomethyl)benzyl]acetamidine (1400W)) [65, 400] or in iNOS deficient mice [25, 291] reduces 'excessive' NO• production during reperfusion and attenuates inflammatory cell tissue infiltration, muscle fiber necrosis and muscle contractile functioning impairments, and thus minimizes ischemia-reperfusion injury. Thus, the increased expression of iNOS in reperfused skeletal muscle potentially contributes to its pathological state [296].

MPO is a major source of tissue injury during ischemia-reperfusion injury. Interestingly, the increase in MPO activity and loss in muscle viability are ameliorated with a NOS inhibitor (nitro-iminothyl-L-orthine) in experimental animal studies [277]. However, other experimental animal studies failed to find any protective benefit of NOS inhibitors (L-NMA and L-NAME) on reperfused skeletal muscle MPO activity when injected immediately before (10 minutes) reperfusion [324]. While both MPO and NOS

contribute to muscle dysfunction following ischemia-reperfusion injury, there appears to be discrepancies in the literature regarding the relation between MPO and NOS during ischemia-reperfusion conditions.

Ischemia-Reperfusion Antioxidants

Probably the greatest support for the hypothesis that oxidative stress causes muscle injury during reperfusion is based on the ability of antioxidants to minimize the reperfusion injury. The increase in vascular permeability that precedes post-ischemic muscle damage is largely ameliorated with antioxidant therapy (i.e., allopurinol, catalase, superoxide dismutase, or dimethyl-sulfoxide (DMSO)) [190, 270] or iron chelator or iron binding protein (i.e.,deferoxamine, apotransferrin) [336]. Antioxidant therapeutic interventions (superoxide dismutase, mannitol and dimethyl thiourea) in experimental animals subjected to ischemia-reperfusion have successfully ameliorated necrosis [377], injury [321] and improved hindlimb muscle function [238, 349]. Furthermore, antioxidant therapy (N-acetyl cysteine and DMSO) has also decreased systemic biomarkers representative of muscle damage (i.e., creatine kinase) and neutrophil infiltration [180].

Neutrophil infiltration is associated with MPO activity [337]. Interestingly, various interventions have successfully decreased MPO in reperfused skeletal muscles. For example, antioxidants (superoxide dismutase, allopurinol, catalase, dimethyl thioreau, DMSO), treatment with an iron chelator (deferoxamine) [324] or the depletion of white blood cells [310] have all been found to ameliorate MPO activity. Moreover, reducing MPO by depleting white blood cells also decreased muscle oxidative stress and necrosis in the reperfused limb muscle [310].

Vitamin E therapy has been successfully demonstrated to be beneficial with regards to ameliorating the oxidative stress response to ischemia-reperfusion insult in mice [13] or in humans undergoing aortic aneurysm surgery [113, 263]. Eight-days of oral vitamin E therapy (600 IU dl-α-tocopheryl acetate) significantly reduced muscle (quadriceps) oxidative stress (i.e., MDA), neutrophil infiltration, preserved myofibril patterns (i.e., myofibrils appeared properly arranged and no disruption of Z-discs), and systemic biomarkers of oxidative stress (i.e., MDA) and muscle damage (i.e., creatine kinase) in elderly individuals following the ischemia-reperfusion conditions elicited during aortic reconstructive surgery [113, 263]. Similarly, oral vitamin C supplementation prior to ischemia-reperfusion significantly preserved muscle function (twitch and tetanus) and reduced MPO activity in reperfused skeletal muscle [169, 170]. Additionally, intravenous infusion of α-tocopherol (100 mg/kg/hr) in rats also significantly reduced systemic biomarkers of oxidative stress (i.e., MDA) and muscle damage (i.e., creatine kinase) and local biomarkers of oxidative stress (i.e., MDA) during reperfusion [45]. Furthermore, in mice weekly intraperitoneal injections of vitamin E (α-tocopherol 60mg/kg of body weight) ameliorated the increase in oxidative stress (i.e., decrease in gluathione) in the soleus muscle subjected to 90 minutes of ischemia and 60 minutes of reperfusion [13]. Ascorbic acid administered by intravenous infusion (80 mg/hour) during ischemia (4-hours) and reperfusion (1-hour) in rabbits preserved muscle integrity by minimizing ultrastructural damage (i.e., mitochondrial swelling, Z-line streamlining and dilated sarcoplasmic reticulum) and ameliorated the increase in blood creatine kinase during reperfusion [52]. Interestingly, allopurinol administration produced no protective effect on systemic or local biomarkers of muscle damage or on MPO in reperfused skeletal muscle [52]. Thus, both oral and intravenous infusion of

vitamins E and C ameliorated systemic and local biomarkers of oxidative stress and muscle damage during reperfusion.

Ischemia-Reperfusion Inflammation

Muscle subjected to ischemia-reperfusion injury also display increased infiltration of inflammatory cells [214, 392]. Interestingly, the increased infiltration into the skeletal muscle parallels the increase in circulating neutrophils [112] and is inversely correlated with muscle function in rats following ischemia-reperfusion injury [374]. Reperfusion-damaged muscle in experimental animals also display an increase in MPO activity [52, 57, 189] which is positively correlated with ischemia duration (1-3 hours) [335]. The progressive increase in MPO activity during reperfusion parallels the increase in oxidative stress (i.e., decrease in muscle glutathione) [175, 337], muscle damage [52] and necrosis [93], and provides further support for a source of inflammatory derived oxidative stress and muscle damage during reperfusion [397].

Ischemia-Reperfusion Inflammatory Cytokines

TNF-α is a pro-inflammatory cytokine synthesized by macrophages and monocytes [211], among other inflammatory cells (i.e., natural killer cells, $CD8^+$, Th1, etc.), and has been prominently linked to the pathophysiology of skeletal muscle. Human skeletal muscles express TNF-α under resting conditions [282]. Experimental animal studies demonstrate that increasing systemic TNF-α causes skeletal muscle injury [117]; while TNF-α (and IL-6) are elevated in humans with age [62, 246, 320, 373] and chronic heart failure [200, 257]; two conditions associated with skeletal muscle atrophy and weakness. Furthermore, TNF-α may contribute to muscle injury by initiating or propagating oxidative stress [205, 206, 209], accelerating muscle atrophy [110, 127,

213], impairing muscle function (i.e., weakness) [301, 384] and promoting neutrophil and macrophage accumulation in muscle [275]. Furthermore, it also appears that the deleterious effects of TNF-α on muscle dysfunction are mediated by oxidative stress [206], which makes amelioration of oxidative stress an attractive therapeutic alternative to minimize muscle dysfunction. Therefore, we tested the role of antioxidant supplementation on oxidative stress, TNF-α and muscle dysfunction (i.e., atrophy and weakness) following an ACL injury and surgery.

IL-6 is also expressed in resting human skeletal muscle [282]. Systemic IL-6 levels also increase during reperfusion following ischemia [152, 324] and may contribute to the increase in muscle atrophy [135]. However, the pathophysiological significance of IL-6 on muscle atrophy and weakness following ACL surgery is unknown.

Fortunately, pro-inflammatory cytokine production is reciprocally regulated and balanced by anti-inflammatory cytokines. In patients undergoing coronary artery bypass grafting that were subjected to cardiopulmonary bypass, a procedure that increases circulating pro-inflammatory cytokines and oxidative stress, Deblier et al. [75] observed an increase in IL-10 at the end of cardiopulmonary bypass and shortly thereafter, but IL-10 returned to pre-surgery levels within hours. Interestingly, following myocardial ischemia-reperfusion injury in humans, the increase in serum IL-10 correlated with MDA and 4-hydroxynonenal (4-HNE) [91]. IL-10 null mice display an increase in TNF-α, nitrate/nitrate and neutrophil infiltration in reperfused heart [396]; whereas in an isolated-perfused mouse liver system, addition of N-acetylcysteine and allopurinol to the perfusate ameliorated the hepatic increases of IL-10 and TNF-α [197]. Furthermore, IL-10 treatment (intraperotineal) of mice attenuated the ischemia-

reperfusion induction of oxidative stress (i.e., the increase in thiobarbituric acid reactive substances (TBARS) and protein carbonyl content, and reduction in glutathione) in the kidney [179]. Thus, the aforementioned findings suggest that IL-10 increases during reperfusion, which may be mediated by either pro-inflammatory cytokines or oxidative stress.

Limb Immobilization Following Anterior Cruciate Ligament Surgery

Another major pathological event experienced by individuals having ACL reconstructive surgery is limb immobilization post-ACL surgery. Several studies have described the structural, functional, and biochemical properties of skeletal muscle following limb immobilization in experimental animals, but the pathogenesis of skeletal muscle atrophy, necrosis, and function of skeletal muscle following limb immobilization in humans remains relatively unknown.

Muscle Morphology, Structure and Function

Muscle atrophy is characterized by wasting or diminution of muscle size. Muscle atrophy is a common consequence of a variety of muscle disuse and disease states (i.e., limb immobilization, mechanical ventilation, cachexia, cancer, bed rest, hindlimb suspension, etc.). Muscle atrophy, as determined from muscle fiber cross-sectional area via muscle biopsies [137] and thigh circumference measurements [22], is significant as early as 1-week post-ACL surgery and may persist for extended period of time, sometimes years, as determined from magnetic resonance imaging [17]. Furthermore, muscle atrophy as determined from muscle fiber cross-sectional areas obtained from muscle biopsies is still apparent following ACL surgery and 5-weeks of limb immobilization with the knee in a 10-15 degree flexion angle [137]. Similarly,

measurements on muscle fiber cross-sectional area demonstrate that muscle atrophy is apparent 6-weeks post-ACL surgery following limb immobilization (1 week of no cast but non-weight bearing followed by 5 weeks of a closed cast from the groin to the ankle in a 45 degree knee flexion angle) [22]. Other measurements of muscle atrophy (e.g. thigh circumference, computed tomography, magnetic resonance imaging) following ACL surgery have also demonstrated a similar atrophy response. Decreases in thigh circumference [122, 223, 260, 264], muscle cross-sectional area [22, 382] and thigh cross-sectional area [98, 148] have been observed from 3-weeks to 6-months post-ACL surgery. Thus, the muscle atrophy response to ACL surgery is well documented using a variety of measurements, and occurs as early as 1-week post surgery.

Deficits in quadriceps strength prior to (i.e., pre-surgery) [239, 240] and following (i.e., post-surgery) [215, 340] ACL reconstructive surgery are prevalent. Furthermore, following ACL surgery quadriceps torque deficits (i.e., isokinetic testing at various degrees per second) have been demonstrated to persist for years compared with the contra-lateral, non-injured limb [17, 21, 309, 358]. Maintaining quadriceps size (i.e., minimizing atrophy) and function (i.e., minimizing weakness) is a critical factor in the differential responses to ACL injuries [387]. It has long been thought that muscle atrophy was one of the prime mediators of the deficits in muscle function following ACL surgery. However, in ACL deficient patients (i.e., individuals who have suffered from an ACL injury but elected not to have treatment or surgery) evidence suggests that there is a lack of correlation between the cross-sectional area of the quadriceps femoris muscle group (as determined from computed tomography), morphological measures, and muscle strength in ACL deficient patients [223]. These findings would suggest that muscle atrophy is not the sole mediatory of muscle weakness in ACL deficient patients.

However, evidence in individuals who elected to receive surgical treatment to repair the ruptured ACL suggest that the loss in muscle function of the quadriceps femoris muscle group 4-weeks post ACL surgery is attributed to a reduction in force producing capacity within the muscle as determined from supra-maximal stimulation data during a maximal isometric (60 degrees knee flexion) voluntary contraction [340]. Conversely, this finding may be challenged by a more recent finding at <12 months post-ACL surgery that indicate the muscle torque per unit volume is significantly lower than control subjects; suggesting torque deficits are not attributed to atrophy (i.e., unit volume) [187]. It is plausible that the deficits in muscle function (and the mechanisms contributing to those deficits) following an ACL injury may be different between those individuals who elected to have surgery compared with those who did not have surgery; and that mechanisms contributing to strength deficits may be different at different time points post-surgery.

Although research pertaining to limb weakness following an ACL injury and surgery has been predominately biomechanically oriented, two recent investigations provide innovative data regarding the early recovery of strength following ACL reconstruction. Gerber et al. [123] studied the influence of eccentric cycling on the early strength recovery of an injured ACL limb, and provides compelling data illustrating a significant improvement in the recovery of quadriceps isokinetic (60º/s) strength by 15 wk post-ACL surgery following only 12 wks of progressive eccentric cycling compared to their standard institutional ACL-rehabilitation protocol. Traditionally, eccentric contractions are linked to muscle damage at the cellular level [288] with subsequent cytokine [339, 361] and oxidative stress responses [67, 236]. Further, ROS/RNS mediate cytokine-derived signaling pathways in experimental animal [347] and cell culture [213] studies,

suggesting, a certain level of ROS/RNS or cytokines, or both, following progressive and repeated-bouts of eccentric cycling may be beneficial with regards to the strength recovery or adaptation of an injured ACL limb following surgery, rather than deleterious. Clearly, future studies examining the signaling pathways and the balance between various cytokines, oxidative stress and antioxidants relative to the strength recovery following an ACL injury and surgery are of clinical and scientific importance.

In addition to the eccentric cycling rehabilitation program-intervention, a recent study investigated the influence of a post-physical therapy protein supplement on the early recovery of quadricep strength following an ACL injury and surgery [148]. Post-physical therapy supplementation (36 sessions) consisting of protein plus carbohydrates induced a greater increase in peak isokinetic strength by 12 wks post-ACL surgery compared to individuals receiving either isocaloric-carbohydrate or placebo supplements [148]. This finding suggests that a protein supplement consumed post-physical therapy facilitates the recovery of force production following an ACL injury and surgery.

Although the studies described by Gerber et al. [123] and Holm et al. [148] provide intriguing data regarding potential mechanisms responsible for muscle weakness, a major hypothesis for weakness pertains to an attenuated neurological loop following an ACL injury [341]; specifically, lower feedback from the injured ACL which reduces the recruitment of high threshold motor units. In addition to pre-surgery data provided by Urbach et al. [369], Konishi and colleagues provide evidence indicating an impaired neurological loop prior to [186] and following [185] ACL surgery. This impairment likely involves the non-injured limb as well [188], but in contrast to the injured limb, the non-

injured limb eventually (≥ 18 months post-surgery) recovers [184]. This finding underscores the importance that strength measurements of the injured, or reconstructed limb, may be underestimated if the non-injured limb is used as a reference.

Inflammation

Knee joint inflammation is a common consequence of an ACL injury and surgery. Controlling knee joint inflammation is an important determinant of improving the functional recovery of the surgically repaired ACL leg [385]. Unfortunately, while knee joint inflammation is highly recognized following ACL injuries and surgery, the systemic and local (i.e., muscles subjected to both ischemia-reperfusion injury and limb immobilization) inflammatory responses to ACL surgery are currently unknown. Understanding the systemic and local inflammatory response to ACL surgery would provide the knowledge required to improve recovery from ACL surgery since inflammation is a powerful mediator of muscle mass and function [298]. More specifically, understanding the inflammatory cytokine response to ACL injuries and the recovery from surgery would provide invaluable knowledge since inflammatory cytokines can aggravate muscle dysfunction [298].

Oxidative Stress

While oxidative stress increases immediately following tourniquet removal during ACL surgery [319, 388], it is currently unknown if there is an increase in oxidative stress during the limb immobilization phase post-ACL surgery. Similar to the inflammatory response, understanding the systemic and local consequences of oxidative stress prior to, during and following ACL surgery would provide important scientific knowledge

regarding the rehabilitation from ACL surgeries because of the deleterious influence of oxidative stress on muscle mass and weakness.

Limb Immobilization or Disuse

Limb Immobilization or Disuse Muscle Morphology, Structure and Function

Experimental animal studies have demonstrated that muscle atrophy begins within 6 hours of limb immobilization [43] and becomes readily apparent after only 24 hours [12]. Studies in both experimental animals (i.e., rats, mice, and rabbits) [41, 42, 168, 262, 329, 357] and humans [161, 356] have established the detrimental consequences of muscle atrophy (i.e., decreases in muscle mass, volume, and fiber cross-sectional area) following various durations of limb immobilization (3-days to10-weeks).

Muscle mass is controlled by the balance between protein synthesis and degradation [160]. For example, immediately following the onset of disuse, there is a rapid decrease protein synthesis that contributes to the early protein loss or atrophy [357]. However, this rapid decrease in protein synthesis plateaus or levels off relatively quickly and is followed by a pro-longed increase in protein degradation [357]. Although a decrease in protein synthesis contributes to atrophy, it arguably appears that an increased rate of protein degradation is the major contributor to muscle atrophy in both experimental animal [18, 161, 253, 357] and human studies [370] beyond the early onset of muscle disuse. In particular, the ubiquitin-proteasome system has been identified as a prime mediator of protein degradation and muscle atrophy in both experimental animal [18, 253] and human studies [161, 370]. Limb immobilization causes an increase in mRNA of the E3 ligases (MuRF1 and MAFbx) associated with

the ubiquitin-proteasome pathway [38]. The increase in various components of the ubiquitin-proteasome pathway are likely a function of oxidatively damaged proteins that are recognized by the ubiquitin-proteasome pathway [73] or by oxidative stress causing their upregulation [208]. Whether it is the former or the latter, experimental human and animal studies provide evidence that an increase in protein degradation contributes limb immobilization or disuse atrophy.

Experimental animal studies demonstrate that during limb immobilization there is an increase in muscle fiber necrosis [11, 168, 245] and distinct ultrastructural damage (i.e., Z-line streamling and myofibrillar disorganization) [165, 167, 199, 247]. Pathological alterations within rat skeletal muscle are apparent after only 24 hours of limb immobilization (i.e., mitochondrial swelling, edema, etc.), and the severity of these alterations, as well other pathological characteristics (i.e., tissue inflammation, necrosis, scalloped sarcolemma, etc.) increase with limb immobilization duration [12]. Concomittant with the pathological increases in muscle necrosis and ultrastructural damage during limb immobilization is an increase in systemic biomarkers representative of muscle damage (i.e., creatine kinase) [167, 199, 245]. Thus, limb immobilization causes an increase in muscle pathology characteristics that can be identified both locally and systemically.

Muscle performance (i.e., isometric strength, peak and total isokinetic work) of the atrophied muscle following limb immobilization is significantly compromised in both experimental human [161, 356] and animal studies [44, 109, 168, 390, 391]. Unfortunately, the power output response to limb immobilization or ACL surgery remains unknown and is of importance since it is a key determinant in muscle function.

Limb Immobilization or Disuse Oxidative Stress

Over the past 15 years oxidative stress has been receiving increasing attention with regards to its deleterious influence on a variety of muscle disuse and disease states that cause muscle atrophy and weakness. Experimental animal studies demonstrate that there is an increase in systemic biomarkers of oxidative stress (i.e., increase in TBARS and decrease in glutathione) during limb immobilization [219]. Additionally, in experimental animals, local biomarkers of oxidative stress (i.e. increased TBARS, decreased glutathione, increased glutathione disulfide, nitrotyrosine, hydroxynonenal (HNE), dichlorohydrofluorescein oxidation) increase in muscles subjected to limb immobilization [181, 183, 218, 219, 325-327] and hindlimb suspension [18, 156, 196, 234, 328, 350]. One potential source of oxidative stress during limb immobilization is MPO, which has been found to increase in immobilized muscle in mice [12].

Recently, in hindlimb-suspended mice, Suzuki et al. [350] provide original and innovative data illustrating the importance of NO• on the induction of skeletal atrophy. As mentioned previously, NO• is produced from 3 different NOS enzymes. In skeletal muscle, nNOS is complexed with dystrophin, a cytoskeletal protein that supports the myofilament lattice and is absent in various muscular dystrophies (i.e., Duchene, Beckers, etc.). Suzuki et al. [350] found following hindlimb suspension that nNOS dissociated from dystrophin (or the sarcolemma) and translocated to the cytoplasm and increased the production of NO• in atrophying muscle.

Although data supporting or refuting oxidative stress as a mediator of muscle atrophy in humans is minimal and subject to debate [27, 28, 304]; recently, Levine et al. [203]

provide compelling data identifying a glutathione decrease (i.e., increase in oxidative stress) in atrophied-diaphragm fibers in mechanically ventilated (i.e., disuse) humans.

Limb Immobilization or Disuse and Antioxidants

Experimental animal studies demonstrate that vitamin E therapy (intraperitoneal injections between 30 mg/kg/day to 60 mg/kg twice weekly) reduces muscle oxidative stress (i.e., prevents the increase in TBARS and the decrease in glutathione) and minimizes muscle atrophy (i.e., alleviates the decrease in muscle weight or muscle fiber diameter) during limb immobilization [14, 181, 182]. Infusion of Trolox (priming dose of 20 mg/kg followed by a constant rate of 4 mg/kg per hour), a water-soluble vitamin E analog, significantly ameliorated deficits in muscle performance, the elevation of 20S proteasome activity and protease activity during mechanical ventilation [35]. However, other experimental animal studies [178] failed to indicate any positive benefit of antioxidant therapy (α-tocopherol, 30 mg/kg intraperitoneal) on preserving muscle mass or muscle function following hindlimb suspension in rats. Similarly, Ikemoto et al. [157] failed to find any antioxidant benefit of vitamin E supplementation (intragastrically delivered α-tocopherol 1.5 or 15 mg/rat) during limb immobilization.

Interestingly, nNOS-null mice and nNOS inhibition (with 7-nitroindazole) muscle atrophy following hindlimb suspension in mice [350]. Thus, it is likely that NO• (or nitrative stress) also mediates skeletal muscle atrophy following limb disuse. This regulation may be mediated through the regulation of various transcription factors (i.e., forkhead box O (Foxo)) and the up-regulation of various proteolytic pathways. However, whether nitrative stress exists during muscle atrophy in humans is unknown.

Limb Immobilization or Disuse and Inflammatory Cytokines

Immobilized rat muscles display an increased presence of inflammatory cytokines (i.e., TNF-α) [399]. In immobilized muscle, significant increases in protein carbonyl content and iNOS have also been documented [399]. Thus, limb immobilization causes an increase in inflammatory cytokine expression within the immobilized muscle; but only limited data is available, especially in humans.

Summary

The ACL is the most commonly injured ligament in the knee. Despite advances in both orthopedic surgery and physical therapy, immediate (1-weeks post-ACL surgery) and persistent (years) atrophy and weakness continue to challenge the rehabilitation to ACL surgery. Identifying the pathophysiological mechanism(s) that contribute to muscle dysfunction following ACL surgery would allow us to design future therapeutic interventions intended to accelerate recovery following surgery.

While the general inflammatory response to ACL injuries and surgery is widely appreciated, recent data in humans during ACL surgery demonstrates that there is also a significant increase in oxidative stress immediately following tourniquet removal. Interestingly, inflammation is a common source of oxidative stress and may be the source of oxidative stress and muscle dysfunction following ACL surgery. Furthermore, inflammatory cytokines induce oxidative stress and induce deleterious effects on skeletal muscle that appear to be mediated by the oxidative stress. Experimental animal studies demonstrate that oxidative stress contributes to muscle atrophy and weakness, which are ameliorated with various antioxidant therapies. The basic

scientific knowledge acquired in experimental animal studies regarding the influence of oxidative stress on muscle dysfunction has not been previously applied in humans, and specifically, in humans recovering from ACL reconstructive surgery, who suffer from both muscle atrophy and weakness. The purpose of this project was to identify the role of the supplementation of vitamins E and C upon the recovery from an ACL injury and surgery on muscle dysfunction.

CHAPTER 3. MODULATION OF INFLAMMATION BY VITAMIN E AND C SUPPLEMENTATION PRIOR TO ANTERIOR CRUCIATE LIGAMENT SURGERY

Tyler Barker, Scott W. Leonard, Roy H. Trawick, Carl R. Kjeldsberg, Thomas B. Martins, Harry R. Hill, Maret G. Traber

Free Radical Biology and Medicine (2009), 46; 599-606
Society for Free Radical Biology and Medicine
Elsevier
11830 Westline Industrial Dr.
St. Louis, MO, USA 63146

Abstract

Muscle atrophy commonly follows anterior cruciate ligament (ACL) injury and surgery. Pro-inflammatory cytokines can induce and exacerbate oxidative stress, potentiating muscle atrophy. The purpose of this study was to evaluate the influence of prior antioxidant (AO) supplementation on circulating cytokines following ACL surgery. A randomized, double-blind, placebo-controlled trial was conducted in men undergoing ACL surgery, who were randomly assigned to either: 1) AO (150 mg RRR-α-tocopheryl acetate (200 IU) and 500 mg ascorbic acid), or 2) matching placebos (PL). Subjects took supplements twice daily for two weeks prior to and up to 12 wk after surgery. Each subject provided five blood samples: 1) baseline (Bsl, prior to supplementation and ~2-wks prior to surgery), 2) pre-surgery (Pre), 3) 90-min, 4) 72-h and 5) 7-d post-surgery. Following surgery, inflammation and muscle damage increased in both groups, as assessed by increased circulating IL-6, C-reactive protein and creatine kinase. During AO supplementation, plasma α-T and AA increased and γ-T concentrations decreased significantly ($P < 0.05$). At 90-min the AO group displayed a significant decrease in AA, an inverse correlation between AA and (interleukin) IL-8 ($r^2 = 0.50$, $P < 0.05$), and a significantly lower IL-10 response than that of the PL group. IL-10 was significantly elevated at 90-min and 72-h in the PL group. In summary, our findings show that circulating inflammatory cytokines increase and AO supplementation attenuated the increase in IL-10 in patients post-ACL surgery.

Introduction

The anterior cruciate ligament (ACL) is one of four major ligaments in the knee that provides stability and strength to the knee joint. Immediate (i.e., 1-wk post-ACL surgery) and persistent (i.e., months to years) leg muscle atrophy commonly follows an ACL injury and surgery [17, 22, 97, 307, 309]. Increases in both circulating oxidative stress markers [319, 388] and synovial fluid pro-inflammatory cytokines [401] have been observed following ACL surgery. Importantly, pro-inflammatory cytokines induce oxidative and nitrative stress [1, 143] and mediate muscle atrophy or proteolysis [78, 127, 128, 212].

Following acute disease, trauma, ischemia-reperfusion injury, or limb remobilization, the induction of a inflammatory cytokine cascade may initiate both circulating and local inflammatory responses [88]. Pro-inflammatory cytokines (i.e., TNF-α, IFN-γ, interleukin (IL)-1β) promote inflammation, whereas anti-inflammatory cytokines (i.e., IL-4, IL-5, IL-10, and IL-13) are up-regulated by and suppress the activity of pro-inflammatory cytokines [88].

The inflammatory response may lead to muscle atrophy. Elevated circulating pro-inflammatory cytokines are related to decreased muscle mass in a variety of human conditions (i.e., aging, COPD, heart failure, etc.) [362, 372, 373]. Other evidence supports the observation that pro-inflammatory cytokines induce muscle atrophy [78], which may be mediated in part by oxidative stress [212].

The regulation of inflammation is an active area of investigation. Anti-inflammatory cytokines provide endogenous protection against pro-inflammatory cytokines. Moreover, there are adverse consequences to taking anti-inflammatory medications [330]. Oral antioxidant (AO) supplementation is an attractive therapeutic

alternative because AO may be able to ameliorate the increase in pro-inflammatory cytokines and the deleterious processes that they potentiate.

Vitamins E (α-tocopherol (α-T)) and C (ascorbic acid (AA)) are two of the major dietary AO that ameliorate oxidative stress [115, 363]. Oxidative stress is commonly described as the over-production (relative to the level of antioxidants) of reactive oxygen (ROS) and nitrogen species (RNS) that elicit protein, lipid and nucleic acid damage [138]. Vitamin E supplementation also attenuates the elevation in pro-inflammatory cytokines in humans [80-82] while minimizing muscle atrophy following limb immobilization [14] and hindlimb suspension [328] in experimental animal studies. Vitamin C recycles vitamin E [47, 139] and is highly concentrated in neutrophils which aids in the protection against the inflammatory-derived respiratory burst [202]. In addition, vitamin C possesses the ability to reduce the secretion of an anti-inflammatory cytokine (i.e., IL-10) from isolated human peripheral blood mononuclear cells [34].

We hypothesized that AO supplementation prior to ACL surgery would attenuate the increase in inflammatory cytokines. Therefore, the purpose of this study was to evaluate the influence of combined vitamin E and C supplementation on the circulating inflammatory cytokine response following ACL reconstructive surgery.

Methods

This study was approved by the Institutional Review Boards at both Oregon State University (Corvallis, OR, USA) and Intermountain Healthcare (Salt Lake City, UT, USA). Subjects were informed of the experimental protocol and procedures and provided both written and verbal consent prior to participation. All subjects were recruited from the practice of one orthopedic surgeon. Recreationally active (i.e., physically active at least 3 times per wk and a minimum of 30 min per session prior to the ACL injury) males between 18 and 45 years of age who suffered from an ACL injury that required and voluntarily elected to have reconstructive surgery were recruited for this study. Subjects were excluded from this study if they had a previous knee ligament injury or other lower extremity musculoskeletal injuries that required the use of crutches for more than 3 consecutive weeks, previous or current history of skeletal muscle pathologies**,** cardiac or peripheral cardiovascular system abnormalities, deep venous thrombosis or pulmonary embolism, clotting disorders, coronary artery disease, peripheral vascular disease, stroke or cancer. Subjects were also excluded if they were currently using warfarin or other anti-coagulants, and suffered from impaired liver or kidney function.

Study Design and Protocol

This study consisted of a double-blind, placebo-controlled, randomized design. Subjects were randomly assigned to one of two oral-supplementation treatment groups: (1) AO (150 mg RRR-α-tocopheryl acetate (200 IU) and 500 mg AA twice daily, n = 10), or (2) matching placebo (PL; 313 mg soybean oil, 110 mg gelatin, 10 mg

water, 7.5 mg beeswax, 8.5 mg carob, 5 mg lecithin twice daily, n = 9) (Carlson Laboratories, Arlington Heights, IL). Supplementation began the day after injury assessment in the physicians' clinic (~2-wk prior to ACL surgery) and concluded upon subject clearance to begin more intense physical activity (~12-wk post-surgery). It should be noted that for the purpose of this study, only the data through the first week post-surgery, which includes tourniquet-induced ischemia-reperfusion, limb immobilization and remobilization, is presented while the 12-wk post-surgery data will be presented in a later manuscript that is currently in preparation. Subjects did not take their supplements the day of surgery but resumed supplementation the day subsequent to surgery. Subjects were instructed to take the supplement with a meal, refrain from any other supplements during participation in this study and discontinue any supplements that they may have been taking prior to study enrollment. All but one subject reported previous supplement use, but no subjects were currently taking any supplements upon study enrollment. Furthermore, subjects that reported previous supplement use, reported supplement use on an irregular or non-consistent basis the previous year. Potential subjects that were unwilling to adhere to our protocol or those who reported daily supplement use for the previous year were not enrolled.

Blood Draws and Sample Handling

Each subject provided five fasting blood draws: (1) baseline (Bsl; prior to supplementation and ~2-wk prior to surgery), (2) pre-surgery (Pre; ~90-min prior to surgery), (3) 90-min post-tourniquet removal, (4) 72-h and (5) 7-d post-surgery. Blood was drawn into one 10 cc green-top Vacutainer tube (143 USP units sodium heparin), one 4.0 cc purple-top Vacutainer tube (1 mg x mL^{-1} EDTA), one 4.5 cc light green top

SST Vacutainer and one 4.0 cc gold top SST Vacutainer. Plasma was separated by centrifugation (Fisher Scientific, Centrific, Model 228, Pittsburgh, PA, USA) at 1380 x g for 15 min within 20 min of sample collection. Following separation, plasma was aliquoted into several different cryotubes. Plasma samples were immediately flash frozen in liquid nitrogen and stored at -80°C until day of analysis. Heparinized plasma samples used for ascorbic acid measurements were handled specially (see below).

ACL Reconstructive Surgery

Subjects were initially anesthetized with propofol (Hospira, Inc., Lake Forest, IL, USA) then maintained on desflurane (Suprane, Baxter Healthcare Corp., Deerfield, IL, USA) during surgery. In addition to general anesthesia, 18 of 19 subjects received a local femoral nerve block (naropin, AstraZeneca LP, Wilmington, DE, USA) during reconstructive surgery. A pneumatic tourniquet was applied “as-high-as-possible” on the thigh of the injured limb at an inflation pressure of 300 mm Hg; the duration of tourniquet application was recorded. In the majority of subjects, the ruptured ACLs were arthroscopically repaired with a double-bundle anatomic, auto (semitendinosus)/allo (semitendinosus) graft. One subject had a bone-tendon-bone (BTB) autograft. Grafts were fixed with a Mitek RIGIDfix (DuPuy Mitek Corp, Warsaw, IA, USA) on the femur and a BIO-INTRAfix (DuPuy Mitek Corp, Warsaw, IA, USA) on the tibia. Tension on each graft was initially set at 70 Newton (N). Following 50 full flexion/extension cycles each graft was decreased to 35 N with a Linvatec graft tensioner (Largo, FL, USA) on the double-bundle anatomic surgeries. For the subject that had a BTB, graft tension initially was set at 50 N and then decreased to 35 N following 50 full flexion/extension cycles with the Linvatec graft tensioner.

Following surgery, subjects were non-weight bearing with the knee immobilized in a leg brace (DonJoy, Vista, CA, USA) locked at 10º of knee flexion until their first post-surgery (5.0 ± 0.2 d) visit with the physician. Thereafter, all subjects were allowed to progress with weight bearing as tolerated. Thus, it should be noted that the 72-h blood draw preceded the progression to weight bearing (i.e., remobilization), whereas the 7-d post-surgery blood draw was ~48-h after subjects started to progress with weight bearing.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Subjects were asked to refrain from using any NSAIDs, including aspirin, ibuprofen and naproxen sodium, throughout the duration of the study other than what was prescribed or recommended by the physician (see below). As an alternative, subjects were allowed to use acetaminophen.

Intravenous and Oral Medication

General post-anesthesia pain management consisted of fentanyl (Mylan, Morgantown, WV, USA, 50 µg per IV every 5 min for 10 minutes max), meperidine (Barr, Pomona, NY, USA, 25 mg per IV every 10 min for 20 minutes max) and morphine sulfate (Hospira, Inc., Lake Forest, IL, USA, 2-4 mg IV every 3 hr). Oral pain medication post-surgery consisted of oxycodone with acetaminophen (Endo, Greenville, NC, USA, 5 mg, one dose following surgery) and ketorolac (Toradol™, Ethex, Toluca, Mexico, 15 mg, one dose following surgery). Additional post-surgery general medication consisted of hydrocodone with acetaminophen (Lortab™, Watson, Corona, CA, USA, 7.5 mg, 1 to 2 tablets every 4 hours daily), Celebrex (Pfizer, New

York, NY, USA, NSAID that is highly specific for COX-2; 200 mg twice daily) and one aspirin (325 mg) a day for the next 3 wk post-surgery.

Analytical Procedures

Plasma α- and γ-Tocopherols

Plasma α- (α-T) and γ-tocopherol (γ-T) concentrations (μM) were measured using high-pressure liquid chromatography (HPLC) with electrochemical detection, as described [283]. Plasma α- and γ-tocopherol concentrations were also standardized to plasma lipids (cholesterol + triglycerides; mmol/mol). Plasma lipids were measured at the clinical lab at Intermountain Healthcare (Salt Lake City, UT, USA).

Plasma Ascorbic and Uric Acid

Immediately following the separation of heparinized plasma, an aliquot was acidified (1:1) with 5% metaphosphoric acid (MPA) containing 1 mM DTPA. The sample was then centrifuged (5 min, 15,000 x g; Mini Spin, Eppendorf, Hamburg), the supernatant removed, frozen in liquid nitrogen, and stored at -80ºC until analysis. Ascorbic (AA) and uric acid (UA) plasma concentrations (μM) were measured using paired-ion reverse-phase HPLC coupled with electrochemical detection, as described [232].

Serum Creatine Kinase

Serum creatine kinase (CK; U/L) was measured using VITROS CK Slides which quantitatively determines CK. This measurement was performed in the clinical lab at Intermountain Healthcare.

Plasma Cytokines

A multiplex microsphere-bead array was used to measure a number of circulating inflammatory cytokines (pg/mL) as previously described [229]. Briefly, cytokines were quantitated using a multiplexed sandwich capture assay developed in the ARUP Institute for Clinical and Experimental Pathology (University of Utah, Salt Lake City, UT) using the Luminex Multi-Analyte Profiling system (Luminex, Austin, TX, USA). Monoclonal capture antibodies for human pro- (TNF-α IFN-γ, IL-1β) and anti-inflammatory (IL-4, IL-5, IL-10, IL-13) cytokines, IL-6 (pro- and anti-inflammatory cytokine), and a chemokine (IL-8) were coupled to microspheres (Luminex). The monoclonal antibodies for TNF-α, IL-4, IL-6, IL-8 and IL-10 were purchased from Pharmingen/BD Biosciences (San Diego, CA, USA), while IFN-γ, IL-5, IL-13 and IL-1β were purchased from Biosciences (San Diego, CA, USA).

Standard curves for each cytokine were made using known concentrations of recombinant human cytokine and performed during the same run as the subject's plasma analysis. All incubations were conducted at room temperature on an orbital plate shaker while protected from light. Subjects' EDTA plasma was diluted 1:2 in a 96-well filter bottom micro-titer plate and incubated for 10 min. Following incubation, beads (25 µl) were added to each well after 2 repeated bouts of vortexing (10 sec) and sonication (20 sec). The plate was then incubated for 1-hr, washed 3X by vacuum filtration with phosphate-buffered saline containing Tween 20 (PBST). Then, 100 µl of a mixture of 9 different biotinylated secondary antibodies were added and incubated for 30 minutes. Following washes (3X) with PBST, 100 µl of 5 µg/ml of streptavidin-conjugated R-phycoerythrin (Moss Substrates, Pasadena, MD, USA) were added to

each well. Plates were then incubated for 20 minutes, washed 3X with PBST. Beads were then re-suspended in 150 µl PBST and mixed for 5 minutes.

Micro-titer plates were then placed in a Luminex 100 instrument for analysis. Microspheres pass through a flow cell where dual laser's identify the microsphere and quantitate the amount of analyte bound to the microsphere by measuring the median fluorescence intensity of the reporter molecule (phycyoerythrin). The median fluorescence intensity of the subjects' sample is then converted into pg/ml based on the known concentrations of the standard curve.

Serum High-Sensitivity CRP (hsCRP)

Automated Chemiluminescent Immunoassay (IMMULITE/IMMULITE 1000 Automated Immunoassay, Diagnostic Products Corp., Los Angeles, CA) quantitatively measured serum hsCRP concentration (mg/L).

Statistical Analysis

To achieve a normal distribution of the data, a rank transformation was performed on cytokine and hsCRP data, and a natural log transformation was performed on CK, α- and γ-T, AA, and UA data. Transformations were checked for normality with a Kolmogorov-Smirnov test. Statistical significance of data (cytokines, hsCRP, CK, cholesterol and triglyceride concentrations) was assessed using a two-way analysis of variance (ANOVA) with repeated measures, followed by a Tukey HSD to test multiple pair-wise comparisons. Multiple linear regression analyses were performed to examine the effect of treatment (AO vs. PL) and time (Bsl, Pre, 90-min, 72-hr and 7-d) on plasma α- and γ-T, AA and UA concentrations. Following a

significant multiple linear regression, paired t-tests were performed to determine within group differences across time, and t-tests were performed to determine differences between treatment groups (AO, n = 10; PL, n = 9) at various times. A one-way ANOVA was performed on subject characteristics (i.e., height, body weight, age) and non-repeated measure outcomes (i.e., tourniquet duration, supplementation duration prior to surgery, and day's non-weight bearing post-surgery). Relationships between variables were examined with a Spearman correlation. Data presented as mean ± SEM, while the cytokine data is presented as median (inter-quartile range). All statistical analyses were performed with SysStat software (SigmaPlot 10.0, SigmaStat 3.5, Chicago, IL, USA). Statistical significance was set at a $P < 0.05$ (for correlation coefficients ($df = N – 2$), N = 10 (AO), $r \geq 0.63$; N = 9 (PL), $r \geq 0.67$).

Results

Subject Characteristics

Subject characteristics were not statistically different between the AO and PL groups (Table 1.1). Specifically, age, height and body weights were comparable between treatment groups upon study enrollment. The duration of supplementation prior to surgery was not significantly different between groups ($P = 0.168$). During reconstructive surgery, tourniquet pressure (300 mmHg) was consistent across all subjects. Moreover, the duration of tourniquet application was not statistically different between treatment groups. Following surgery, there were no significant differences in the duration of non-weight bearing activity between AO and PL groups.

With respect to circulating lipid levels, there were no significant differences in serum cholesterol concentrations between the AO (4.49 ± 0.21, 4.55 ± 0.23, 4.12 ± 0.23, 4.40 ± 0.31 and 4.69 ± 0.30 mmol/L at Bsl, Pre, 90-min, 72-h and 7-d, respectively) and PL (4.84 ± 0.33, 5.14 ± 0.41, 4.85 ± 0.38, 4.89 ± 0.44 and 5.03 ± 0.29 mmol/L at Bsl, Pre, 90-min, 72-h and 7-d, respectively) groups. However, surgery did have an impact on circulating cholesterol levels. A significant decrease in cholesterol was observed at 90-min compared with Pre and 7-d serum concentrations (see Table 3.1). In contrast, there were no significant differences in triglyceride concentrations with study time or treatment.

Serum Creatine Kinase

Although the time courses were not statistically different between treatment groups, there was a significant main effect of time on CK ($P < 0.05$, Figure 3.1). CK

concentrations at 72-h were significantly greater than that at Bsl, Pre, 90-min and 7-d. By 7-d, CK returned to pre-surgery concentrations. These data suggest that there is a significant but modest degree of muscular insult during ACL surgery.

Plasma α- and γ-Tocopherol Concentrations

AO supplementation lead to significantly increased plasma α-T concentrations (μM), as well as lipid standardized plasma α-T concentrations (plasma α-T /(total cholesterol + lipids); mmol/mol, treatment x time interaction, $P < 0.05$, Figures 3.2 A and B). At Bsl, there were no significant differences in α-T concentrations between groups. With the exception of lipid standardized α-T concentrations at 72-h compared to that at 90-min, the PL group demonstrated no significant differences over time in plasma or lipid standardized α-T concentrations.

AO supplementation decreased plasma γ-T concentrations, as well as lipid standardized concentrations (significant treatment x time interaction, $P < 0.05$, Figures 3.3 A and B). There were no significant differences in γ-T between treatment groups at Bsl.

Plasma Ascorbic and Uric Acid Concentrations

AO supplementation increased plasma AA concentrations (significant treatment x time interaction, $P > 0.05$, Figure 3.4). At Bsl, there were no significant differences in AA concentrations between treatment groups. Within the PL group, by 7-d post-surgery plasma AA concentrations had decreased significantly relative to Bsl levels. Within the AO group, plasma AA concentrations increased significantly compared with Bsl and relative to the concentrations in the PL group with the exception of 72-h post-surgery.

Interestingly, within the AO group at 90-min post-surgery (i.e., post-tourniquet removal), AA concentrations were decreased ($P < 0.05$) relative to concentrations measured ~90-min prior to surgery.

There were no significant differences in plasma UA concentrations across time (339 ± 14, 350 ± 12, 347 ± 15, 330 ± 16 and 329 ± 14 μM at Bsl, Pre, 90-min, 72-h and 7-d, respectively); or between treatment groups (AO: 357 ± 18, 347 ± 19, 344 ± 23, 323 ± 27, 334 ± 25 μM at Bsl, Pre, 90-min, 72-h and 7-d, respectively, and PL: 318 ± 21, 353 ± 15, 350 ± 19, 337 ± 16, 322 ± 10 μM at Bsl, Pre, 90-min, 72-h and 7-d, respectively).

Circulating Cytokines and hsCRP

Plasma inflammatory cytokines and serum hsCRP concentrations were measured at the various time points. No statistically significant differences were observed across time or between treatment groups in concentrations of TNF-α, IFN-γ, IL-1β, IL-4, IL-5, IL-13 or IL-8 (Table 3.2). Although there were no effects of AO treatment, there were some effects of time on IL-6 and hsCRP concentrations (Table 3.3). Specifically, at 72-h, IL-6 and hsCRP concentrations were significantly elevated and remained elevated at 7-d compared with those at Bsl, Pre and 90-min. Various pro-inflammatory cytokines (i.e., TNF-α, or IL-1β) induce IL-6 production [3, 31, 121, 248], which in turn increases the hepatic production of hsCRP [60, 120]. Although we did not observe a significant change in other pro-inflammatory cytokines, it is likely that there was a transient elevation in other pro-inflammatory cytokines following surgery that mediated the increase in IL-6 production.

In contrast to other cytokines, IL-10 concentrations displayed a significant treatment x time interaction ($P < 0.05$, Table 3.4). Within the PL group, at 90-min IL-10 concentrations were significantly elevated compared with concentrations at the other time points and were also significantly greater than those of the AO group at 90-min. Furthermore, the PL groups' IL-10 concentrations at 72-h were significantly greater than Bsl and Pre. No significant changes were detected in the AO group. These data suggest that AO treatment attenuated the demand to increase the production of IL-10, an anti-inflammatory cytokine that balances and provides protection against the increase in pro-inflammatory cytokines.

Correlation Between Ascorbic Acid and IL-8

Interleukin (IL)-8 facilitates the migration of leukocytes from the circulation to the site of inflammation and injury [88], and is up-regulated by oxidative stress [77, 194] and down-regulated by AO [76]. AA is also a potent plasma AO [115]. Although there were no significant differences in IL-8 across time or between groups, at 90-min we found a significant inverse association between AA and IL-8 in the AO (Figure 3.5 B), but not in the PL group (Figure 3.5 A). This finding suggests that the increase in plasma AA concentration following vitamin C supplementation potentially reduced the migration of inflammatory cells to the site of inflammation or injury.

Discussion

The purpose of this study was to evaluate the influence of AO supplementation on inflammation by measuring circulating biomarkers in subjects with an ACL injury, before and after surgical repair. Vitamin E and C supplementation prior to ACL surgery ameliorated the increase in IL-10 observed at 90-min post-surgery, following tourniquet-induced ischemia. Furthermore, IL-10 remained elevated within the PL group at 72-h compared to pre-surgery levels.

With respect to pro-inflammatory processes, both IL-6 and hsCRP were elevated in all subjects at 72-h and 7-d compared with concentrations before and immediately after surgery (i.e., 90-min). Interestingly, the elevations in inflammatory cytokines (pro- and anti-) and the increase in hsCRP corresponded temporally with an elevation in CK at 72-h post-surgery. Thus, circulating biomarkers provide evidence of a cytokine and muscle insult following ACL surgery. Moreover, AO supplementation ameliorated the endogenous production IL-10 during reperfusion immediately following tourniquet release. These latter findings suggest that the extent of inflammation was attenuated by the by AO supplementation, thus obviating the necessity for the IL-10 excursion.

We found a significant increase in circulating IL-6 and hsCRP during the days (72-h and 7-d) following ACL surgery (Table 3.3). Others have reported increases in local (i.e., synovial fluid) pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) following an ACL injury [56, 147, 158] and surgery [401]. IL-6 production by monocytes, fibroblasts and skeletal muscle is potentiated by an elevation in pro-inflammatory cytokines and oxidative stress [3, 191, 248], with an increase in IL-6 stimulating the hepatic production of hsCRP [60]. Importantly, high circulating levels of

IL-6 are associated with low muscle mass [362, 373] or muscle wasting [372] in various pathophysiological (i.e., COPD, heart failure, etc.) and non-pathophysiological (i.e., aging) conditions in humans. Additionally, muscle atrophy occurs in response to increases in IL-6 [135, 366]. Thus, it is likely that the increase in circulating inflammatory cytokines following ACL injury triggers the early muscle atrophy response to ACL surgery.

Oxidative stress is characterized by the overproduction of various ROS and RNS that elicit nucleic acid, lipid and protein damage [138]. Over the past couple of decades there have been tremendous advancements in our understanding of additional and concomitant deleterious processes regulated by oxidative stress, such as muscle atrophy and weakness [287, 297]. Interestingly, following the tourniquet-induced ischemia during ACL surgery, oxidative stress biomarkers increase [319, 388]. When taken collectively with the findings from the present study, there appears to be both an increase in circulating inflammatory cytokines and oxidative stress following ACL surgery. Thus, these immediate post-surgery signaling events may regulate the degree of muscular impairment following ACL surgery. This suggestion is based on the observation that inflammatory cytokines and oxidative stress both lead to muscle atrophy and weakness [78, 127, 143, 212, 237, 249, 250, 328, 350], two predominant impairments that commonly follow ACL surgery. Furthermore, these findings also provide direction for future therapeutic interventions, specifically AO supplementation since they attenuate the increase in both inflammatory cytokines and oxidative stress.

Although the AO properties of α-T are highly recognized [363], α-T also acts as a anti-inflammatory agent. In a number of randomized, double-blind, placebo-control studies conducted by Devaraj and colleagues [80-82], α-T supplementation for 6-wk (800 mg/d), 3-mo and 2-yr (900 mg/d) significantly lowered circulating pro-inflammatory

cytokines (TNF-α, IL-6) and hsCRP in normal healthy control subjects, subjects with metabolic syndrome, patients with coronary artery disease that were using drug therapy, and in Type 2 diabetes subjects (with and without macro-vascular complications). However, responses to vitamin E supplementation have been inconsistent. Wu et al. [394] failed to find a significant influence of a lower daily dosage of α-T (500 mg/d for 6-wk) on plasma pro-inflammatory cytokines (TNF-α, IL-6) and hsCRP in Type 2 diabetics. This latter finding is similar to our results in ACL surgery patients, albeit at a lower daily dose of α-T (300 mg/d) that was also combined with AA (1000 mg/d) at a substantially shorter supplementation duration (~2-wk prior to surgery and 1-wk thereafter). However, pro-inflammatory cytokines are also balanced by anti-inflammatory cytokines, and therefore, raises the question, does AO supplementation influence the production of anti-inflammatory cytokines?

Interleukin (IL)-10 is an anti-inflammatory that is transcriptionally regulated by nuclear factor-kappa B (NF-κB), among others (i.e., cAMP response element, a glucorticoid response element, etc.) [99, 173, 252], in response to an increase in pro-inflammatory cytokines and oxidative stress. Previously in patients undergoing coronary artery bypass grafting that were subjected to cardiopulmonary bypass (CPB), procedures that cause an increase in circulating pro-inflammatory cytokines and oxidative stress, Deblier et al. [75] observed a increase in IL-10 at the end CPB and shortly thereafter, but returned to pre-surgery levels within hours. In isolated human peripheral white blood cells incubated with vitamin E and C, Bergman et al. [34] found that vitamin C reduced the production of IL-10 in a dose-dependent fashion without influencing the production of TNF-α. In the present study, IL-10 concentrations changed differently over time in the two treatment groups (Table 3.4). Within the PL group, IL-10 was elevated at 90-min and 72-h, with the 90-min value being greater than

that in the AO group. Differences in IL-10 concentrations also corresponded with changes in plasma AA. Specifically, at 90-min post-tourniquet removal, AA concentrations decreased, while IL-10 remained unchanged in the AO group. In contrast, AA was unchanged, while IL-10 was significantly increased in the PL group. Thus, it is plausible that AO supplementation increased the ROS/RNS scavenging capacity and therefore reduced the oxidative stress stimulus to increase IL-10 production. However, further studies are needed to evaluate the IL-10 response following AO supplementation and ACL surgery.

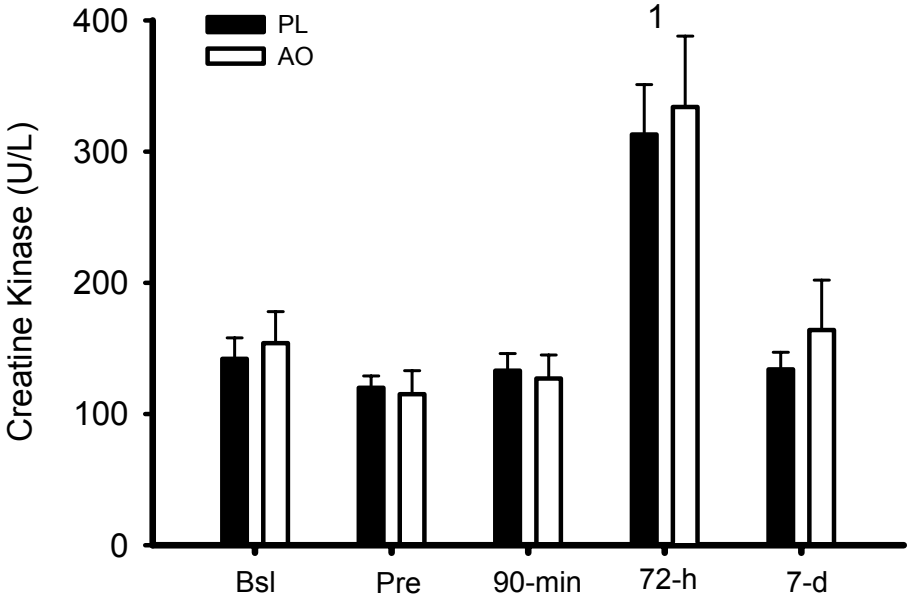
In addition to an association with IL-10, we also observed a significant inverse association between AA and IL-8 (Figure 3.5 B). Interleukin (IL)-8 is a neutrophil chemo-attractant that facilitates neutrophil migration to the site of inflammation and injury, and is up-regulated in response to an elevation in pro-inflammatory cytokines [346] and oxidative stress [76, 77]. IL-8 production is tightly regulated such that the same pro-inflammatory cytokines that stimulate its production also generate IL-10, which inhibits IL-8 synthesis [378, 379]. Based on this negative cytokine-cascade feedback loop, one would anticipate lower IL-8 levels in the PL group because of a significant increase in IL-10 at 90-min compared to that of the AO group. However, there was not a significant correlation between IL-8 and IL-10 in the either PL ($r = 0.45$, $P = 0.20$; data not shown) or AO ($r = -0.10$, $P = 0.76$; data not shown) groups at 90-min. However, a diverse set of experimental studies demonstrate that a variety of AO treatments blunt the increase in IL-8 and neutrophil recruitment [76, 77, 231]. Although there were no significant differences in IL-8 across time or between groups, the observed inverse correlation between AA and IL-8 in the AO group suggests that an increase in circulating AA levels lowers the neutrophil chemo-attractant response. Along similar lines, vitamin E and C supplementation may reduce the infiltration of

neutrophils into skeletal muscle during reperfusion as seen in both human [113, 263] and animal [169, 170] studies, respectively. More recently, Judge et al. [163] found that oxidative stress and neutrophil infiltration mediated by contractile claudication (form of ischemia-reperfusion) were ameliorated with vitamin E and C supplementation in rats. Therefore, it is possible that AO provided protection by lowering IL-8 and the subsequent inflammatory response at the site of knee surgery and the lower limb musculature subjected to tourniquet-induced ischemia-reperfusion injury. However, additional studies are required to examine whether the local (i.e., knee and/or muscle) inflammatory and oxidative stress responses are influenced by AO supplementation.

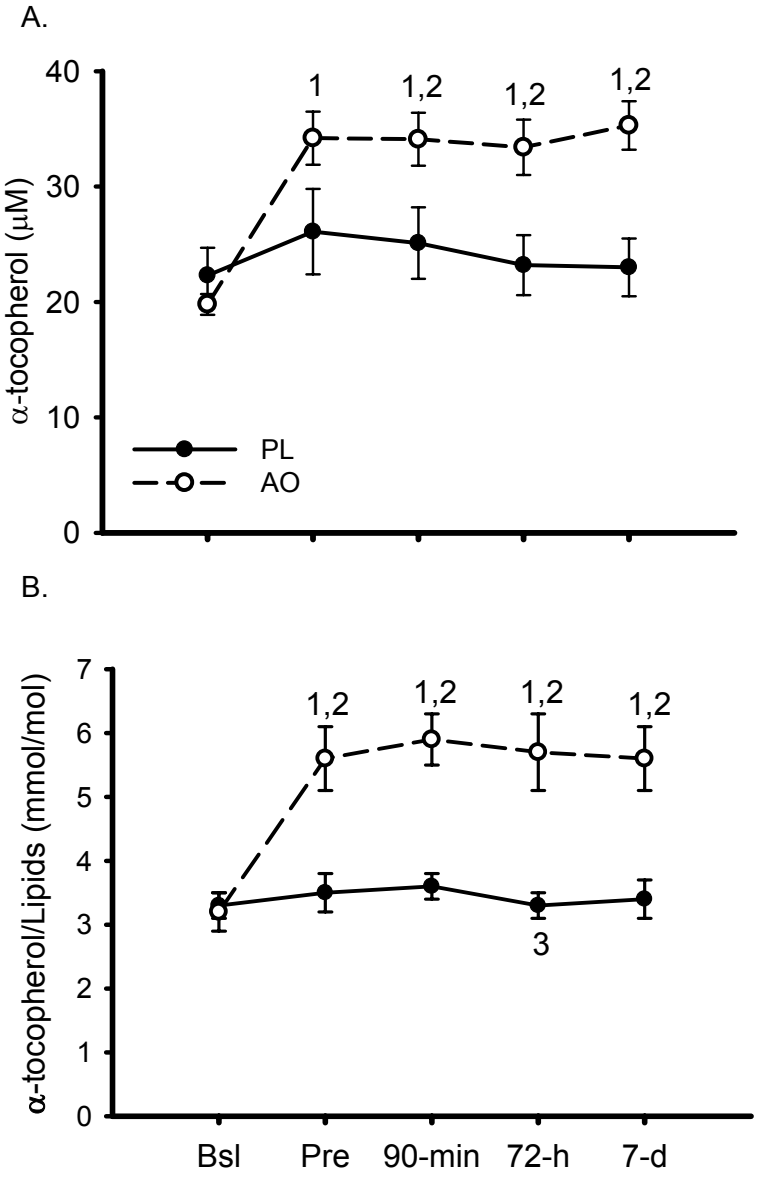
There are some limitations of the present study that require attention. It would have been ideal to perform more frequent blood draws immediately following tourniquet removal during surgery. It is likely that the more frequent blood draws would have identified an increase in other pro-inflammatory cytokines during reperfusion. Another limitation of this study was that subjects were prescribed Celebrex to assist with inflammation and pain control. Celebrex is a NSAID that is specific to cycloxygenase-2 and it is clear that our cytokine data was influenced by the prescribed NSAID supplementation. However, NSAID prescription was consistent and consistently taken across all subjects in the current protocol. Therefore, differences between treatment groups (AO vs. PL) would likely be minimal.

In summary, an increase in circulating inflammatory cytokines was observed following the reconstructive repair of a damaged ACL. The AO supplementation ameliorated the increase in IL-10 that occurred during reperfusion, at 90 min post-surgery. We are currently investigating the prevalence and significance of some of the proposed mediators of muscle atrophy immediately following ACL surgery.

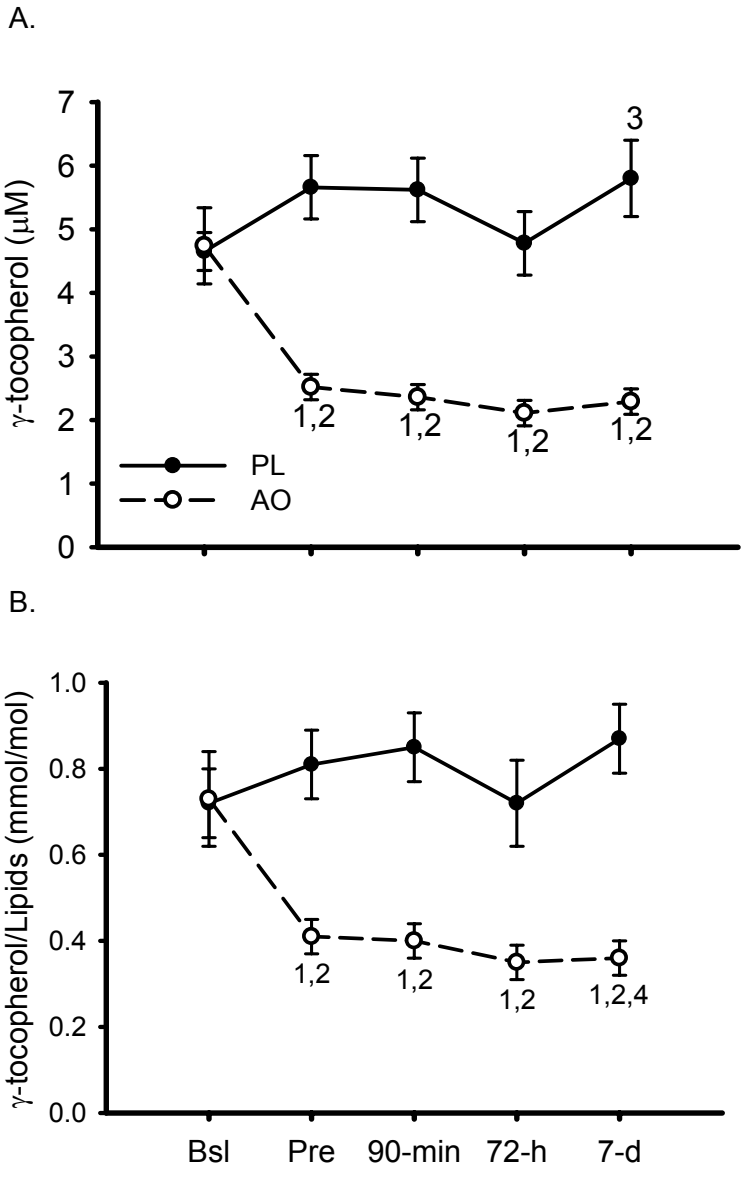
Figures



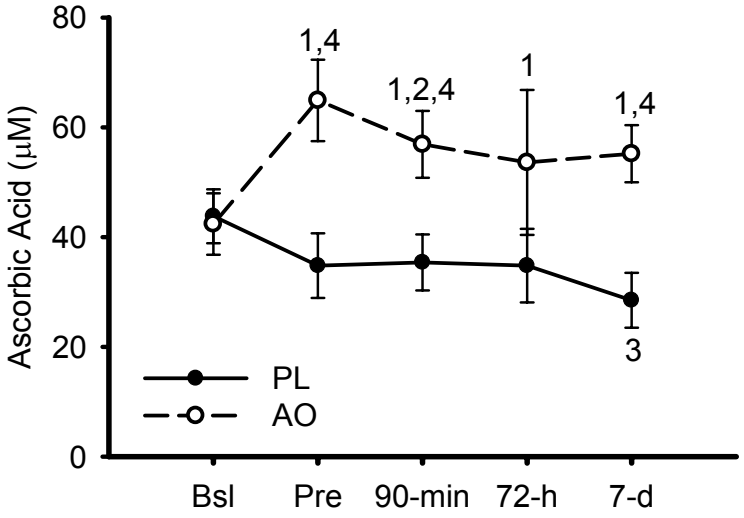
3.1. Serum Creatine Kinase (CK; U/L). Data presented as mean ± SEM (PL n = 9; AO n = 10). [1]Significantly ($P < 0.05$) different from Bsl, Pre, 90-min and 7-d. There were no significant differences in CK between groups (AO vs. PL).



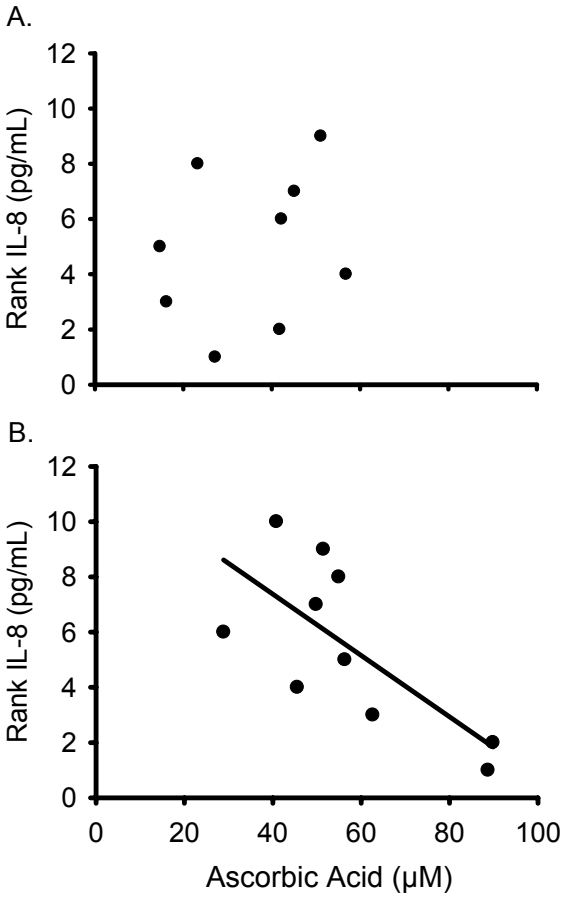
3.2. Plasma (A) α-Tocopherol (μM) and (B) α-Tocopherol/lipids (mmol/mol). Data presented as mean ± SEM. There was a significant ($P < 0.05$) treatment x time interaction for both α-tocopherol and α-tocopherol/lipids. [1]Significantly different from AO Bsl. [2]Significantly different from corresponding PL. [3] Significantly different from PL 90-min.



3.3. Plasma (A) γ-Tocopherol (μM) and (B) γ-Tocopherol/lipids (mmol/mol). Data presented as mean ± SEM. There was a significant ($P < 0.05$) treatment x time interaction for both γ-tocopherol and γ-tocopherol/lipids. 1Significantly different from AO Bsl. 2Significantly different from corresponding PL. 3Significantly different from PL 72-h. 4Significantly different from AO Pre.



3.4. Plasma Ascorbic Acid Concentrations (µM). Data presented as mean ± SEM. There was a significant ($P < 0.05$) treatment x time interaction. [1]Significantly different from AO Bsl. [2]Significantly different from AO Pre. [3]Significantly different from PL Bsl. [4]Significantly different from corresponding PL.



3.5. Correlation Between Ascorbic Acid (μM) and Rank IL-8 (pg/mL) at 90-min in The PL (A) and AO (B) Treatment Groups. Plasma ascorbic acid (μM) correlated with the rank of IL-8 (pg/mL) at 90-min in the AO (B; n = 10; r = 0.71; P < 0.05) but not the PL (A; n = 9; r = 0.24; P = n.s.) group. n.s., non-significant (P > 0.05).

Tables

3.1. Subject Characteristics

	PL	AO	GR
n	9	10	19
Age (yr)	36 ± 3	32 ± 2	33 ± 2
Ht (cm)	174 ± 4	175 ± 2	175 ± 2
Bwt (kg)	89 ± 5	89 ± 7	89 ± 5
Serum Cholesterol (mmol/L)	4.94 ± 0.31	4.45 ± 0.29	4.69 ± 0.10[1]
Serum Triglycerides (mmol/L)	2.07 ± 0.34	1.82 ± 0.32	1.94 ± 0.12
Days supplemented pre-surgery	12 ± 2	16 ± 2	14 ± 1
Tourniquet Duration (min)	61 ± 6	63 ± 5	62 ± 4
Days non-weight bearing post-surgery	5.0 ± 0.4	5.0 ± 0.2	5.0 ± 0.2

Data presented as mean ± SEM. [1]Serum cholesterol at 90-min (4.46 ± 0.23 mmol/L) was significantly ($P < 0.05$) lower than that at Pre (4.83 ± 0.23 mmol/L) and 7-d (4.85 ± 0.21 mmol/L). There were no significant differences in cholesterol between groups (AO vs. PL). GR, pooled (PL + AO) data.

3.2. Plasma Cytokines Were Unaffected by Surgery or Antioxidant Treatment.

	TNF-α	IFN-γ	IL-1β	IL-4	IL-5	IL-13	IL-8
Bsl	2.0 (1.0, 6.1)	3.0 (1.2, 11.1)	5.2 (2.9, 7.9)	1.3 (1.2, 2.0)	2.3 (1.8, 3.9)	3.3 (2.7, 5.9)	11.6 (8.7, 13.7)
Pre	1.9 (1.0, 7.5)	3.5 (1.5, 12.4)	5.6 (2.9, 8.5)	1.3 (1.1, 2.0)	2.4 (1.8, 3.6)	2.7 (1.9, 4.9)	11.1 (7.3, 18.4)
90-min	2.1 (1.0, 6.3)	3.4 (1.0, 11.8)	4.7 (3.0, 7.2)	1.2 (1.0, 1.8)	2.4 (1.5, 3.2)	3.3 (1.2, 4.8)	12.8 (9.4, 19.3)
72-h	1.9 (1.0, 5.1)	3.3 (1.1, 13.3)	5.6 (3.1, 8.6)	1.3 (1.1, 2.1)	2.8 (2.4, 3.5)	2.7 (2.0, 4.7)	14.7 (11.8, 23.4)
7-d	1.8 (1.1, 7.7)	4.2 (1.4, 11.2)	5.2 (2.3, 10.5)	1.3 (1.0, 2.4)	2.5 (1.9, 4.3)	2.8 (2.0, 5.7)	14.3 (10.8, 18.8)

Data presented as median (inter-quartile range). Group (pooled PL and AO) cytokine (pg/mL) concentrations were not significantly different across time or between treatments (PL vs. AO).

3.3. Both IL-6 and hsCRP Concentrations Were Significantly Increased at 72-h and 7-Days.

	IL-6 (pg/mL)	hsCRP (mg/L)
Bsl	11.5 (6.9, 22.4)	3.4 (2.0, 4.6)
Pre	11.4 (8.0, 26.3)	2.5 (1.6, 3.5)
90-min	13.0 (8.5, 32.0)	2.5 (1.5, 3.4)
72-h	30.0 (25.8, 48.6)[1]	36.7 (27.3, 58.7)[1]
7-d	27.4 (16.7, 43.6)[1]	15.7 (12.0, 28.4)[1]

Group (pooled PL and AO) IL-6 and hsCRP concentrations are presented as median (inter-quartile range).
[1]Significantly ($P < 0.05$) different from Bsl, Pre and 90-min; not significantly different between treatment groups.

3.4. IL-10 (pg/mL) Increased Significantly in The PL But Not The AO Group.

Treatment	PL	AO
Bsl	4.6 (4.2, 6.3)	8.1 (4.6, 21.6)
Pre	5.0 (4.6, 6.2)	7.0 (4.3, 24.8)
90-min	22.9 (13.7, 41.4)[1,3]	14.0 (3.6, 24.9)
72-h	7.5 (5.8, 8.2)[2]	9.7 (6.7, 26.1)
7-d	5.7 (4.8, 6.6)	10.8 (5.9, 43.1)

IL-10 concentrations presented separately for PL and AO groups as median (inter-quartile range); significant treatment x time interaction ($P < 0.05$).
[1]Significantly different from Bsl, Pre and 7-d within the PL group.
[2]Significantly different from Bsl and Pre within the PL group.
[3]Significantly different from corresponding AO.

CHAPTER 4. VITAMIN E AND C SUPPLEMENTATION ON MUSCLE DYSFUNCTION FOLLOWING AN ANTERIOR CRUCIATE LIGAMENT INJURY AND SURGERY: BENEFICIAL OR DETRIMENTAL?

Tyler Barker, Scott W. Leonard, Janet Hansen, Roy H. Trawick, Katherine M. Lebold, Thomas B. Martins, Carl R. Kjeldsberg, Harry R. Hill, Ronda Ingram, Graham Burdett, James A Walker and Maret G. Traber

Abstract

Muscle dysfunction (i.e., atrophy and weakness) is a predominant impairment following one of the most commonly injured and surgically repaired ligaments in the knee, the anterior cruciate ligament (ACL). Oxidative stress and pro-inflammatory cytokines mediate muscle dysfunction; while vitamins E and C protect against muscle dysfunction by ameliorating oxidative stress and pro-inflammatory cytokines. The purpose of this study was to test the hypothesis that vitamin E and C supplementation improves recovery following an ACL injury and surgery by attenuating the increase in circulating and local mediators of muscle dysfunction. Twenty-males undergoing elective ACL surgery were randomly assigned to either: 1) antioxidant (AO, vitamins E and C, n = 10), or 2) matching placebos (PL, n = 10). Supplements were taken twice daily with a meal for two weeks prior to and for 3-mo post-surgery. Each subject provided several fasting blood draws, two muscle biopsies from the thigh muscle of the injured limb, and strength measurements on the injured and non-injured limbs prior to and following surgery. Immediately following ACL surgery, there was a significant ($P < 0.05$) increase in circulating oxidative stress marker (i.e., 8-isoprostane-$PGF_{2\alpha}$) and a depression of a pro-to-anti-inflammatory cytokine ratio (i.e., IL-6:IL-10). AO supplementation stabilized ($P < 0.05$) the depression of the IL-6:IL-10 ratio but did not reduce oxidative stress. Furthermore, AO supplementation significantly ($P < 0.05$) increased the infiltration of inflammatory cells into the musculature of the injured ACL limb following surgery. Unlike the PL group, AO takers did not show improved strength recovery of the injured limb. We conclude that although vitamin E and C supplementation induced immunological alterations immediately after surgery that

would be suggestive of a better prognosis thereafter, our data indicates that supplementation was ineffective or possibly detrimental on the recovery of muscle following ACL reconstruction.

Introduction

Skeletal muscle dysfunction (i.e., atrophy and weakness) is a major cause of morbidity in a variety of pathophysiological and non-pathophysiological conditions (i.e., aging, chronic heart failure, chronic obstructive pulmonary disorder, damaging or fatiguing exercise, etc.) [62, 119, 150, 233, 246, 257, 320, 360, 373]. Muscle (i.e., quadriceps) dysfunction is also a predominant impairment that commonly follows an anterior cruciate ligament (ACL) injury and surgery [17, 21, 97, 123, 129, 148, 184, 186, 240, 340]. Although attenuated mechanoreceptor feedback from a ruptured or surgically repaired ACL contributes to muscle weakness [184-186], it is probable that other pathophysiological mechanisms accentuate muscle weakness following ACL reconstruction. Specifically, oxidative stress [10, 48, 55, 146, 249, 250, 348] and pro-inflammatory cytokines [6, 89, 143, 205, 301, 383, 384] induce muscle weakness. However, evidence in humans, especially in a clinical setting, has been equivocal.

Vitamins E [363] and C [58] are potent antioxidants that display immuno-modulatory properties [80, 227, 244]. In elderly humans, low antioxidant status (i.e., low vitamin E) is associated with a decline in physical function [30], whereas a high antioxidant status (vitamins E or C) is associated with a reduction in frailty [36] and an increase knee extension strength [61]. Furthermore, in experimental animals (i.e., rats and mice) vitamin E treatment attenuated muscle atrophy induced by hindlimb unloading [328] and immobilization [14, 182] minimized the induction of pro-inflammatory cytokines (i.e., TNF-α, IL-1β and IL-6) [153]; cytokines that regulate atrophy or pathways that govern atrophy [46, 135, 210]. Although oxidative stress [150, 203, 269] and an increase in pro-inflammatory cytokines [195, 200, 320] are associated

with muscle dysfunction in humans, it is currently unknown if vitamin E and C supplementation ameliorates muscle dysfunction following an ACL injury and surgery.

We hypothesized that vitamin E and C supplementation would minimize muscle dysfunction (i.e., atrophy and weakness) following ACL reconstructive surgery by ameliorating the increase in circulating and local mediators of dysfunction. To address this objective, we conducted a randomized, double-masked, placebo-controlled (i.e., oral vitamin E and C vs. matching placebo) study in patients having ACL reconstructive surgery.

Methods

This study was approved by the Institutional Review Boards at both Oregon State University (Corvallis, OR, USA) and Intermountain Healthcare (Salt Lake City, UT, USA). Subjects were informed of the experimental protocol and procedures and provided both written and verbal consent prior to participation. The study exclusion and inclusion criteria have been published previously [26].

Study Design and Protocol

The description of the experimental design for this study [26] is illustrated in Figure 4.1. Briefly, this study consisted of a double-masked, placebo-controlled, randomized design. Twenty, physically-active (i.e., continuous activity for 30 minutes at least 3 times per week) male subjects were randomly assigned to one of two oral-supplementation treatment groups: (1) AO (n =10, 200 IU vitamin E (50% d-α-tocopherol and 50% d-α-tocopheryl acetate) and 500 mg ascorbic acid (AA) twice daily), or (2) matching placebo (PL, n = 10) (a generous gift from Carlson Laboratories, Arlington Heights, IL, USA). Supplementation began the day after injury assessment in the physicians' clinic (~2-wk prior to ACL surgery) and concluded 3-mo post-surgery. Subjects did not take their supplements the day of surgery but resumed supplementation the day after surgery. Subjects were asked to refrain from any physical activity or therapy 72-h prior to blood draws, muscle biopsies and single-leg strength testing (see below).

Blood Draws and Sample Handling

Each subject provided six fasting blood draws: (1) baseline (Bsl; prior to supplementation and ~2-wk prior to surgery), (2) pre-surgery (Pre; ~90-min prior to surgery), (3) 90-min post-tourniquet removal, (4) 72-h, (5) 7-d and (6) 3-mo post-surgery (Figure 4.1). Blood was drawn from the antecubital vein into one 10 cc green-top Vacutainer tube (143 USP units sodium heparin), one 4.0 cc purple-top Vacutainer tube (1 mg x mL^{-1} EDTA), one 4.5 cc light green top SST Vacutainer and one 4.0 cc gold top SST Vacutainer. Plasma was separated by centrifugation (Fisher Scientific, Centrific, Model 228, Pittsburgh, PA, USA) at 1380 x g for 15 min within 20 min of sample collection. Plasma sample aliquots were immediately flash frozen in liquid nitrogen and stored at -80°C until day of analysis. Heparinized plasma samples used for AA measurements were handled specially (see below).

Percutaneous Needle Muscle Biopsies

Each subject provided two percutaneous needle (CardinalHealth, REF2N2702X; 14G x 11.4cm, McGraw Park, IL, USA) muscle biopsies from the mid-vastus lateralis muscle of the injured limb. The pre-surgery (Pre) biopsy was taken the day of ACL surgery but prior to any surgical procedures. The post-surgery (Post) biopsy was taken following limb immobilization or the non-weight bearing phase the first several days after surgery. The second biopsy was taken 2-3 cm proximal or distal from the Pre biopsy site. The biopsy site was cleaned and sterilized. Local topical (Gebauer Ethyl Chloride, Gebauer Company, Cleveland, OH, USA) and percutaneous

(1% Lidocaine HCL with epinephrine, USP, Xylocaine, 10 mg/mL, AstraZeneca, Wilmington, DE, USA) anesthesia were applied to numb the biopsy site. Biopsy samples were immediately fixed in 10% neutral buffered formalin and embedded with paraffin for later immunohistochemical measurements.

Muscle biopsies from 14 (PL, n = 7; AO, n = 7) of the 20 subjects were measured for fiber cross-sectional areas (FCSA), fast-myosin, myeloperoxidase (MPO), tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) (see below). With regard to the subjects that are not reported, one subject refused the post-surgery biopsy, while the other subjects' biopsy specimens (either Pre or Post, or both) were either small or fragmented.

Single-Leg Testing Protocol

Single-leg peak isometric force measurements were performed on the non-injured and injured limbs at Bsl and 3-mo post-ACL surgery. Single leg peak power output measurements were performed on the non-injured limb at Bsl and 3-mo post-surgery, while peak power output measurements on the injured limb were only performed at 3-mo. We did not want to risk additional knee injury; therefore, we deemed it unnecessary to perform the repetitive contractions associated with the single leg power output measurement prior to ACL reconstructive surgery (see below).

ACL Reconstructive Surgery

General surgical, anesthesia and medication (oral and intravenous) have been previously reported [26]. Briefly, a pneumatic tourniquet was applied “as-high-as-possible” on the thigh of the injured limb at an inflation pressure of ~300 mm Hg; the duration of tourniquet application was recorded. For the majority of subjects, ruptured ACLs were arthroscopically repaired with a double-bundle anatomic, auto (semitendinosus)/allo (semitendinosus) graft. One subject had a bone-tendon-bone autograft. Eleven (PL, n = 4; AO, n = 7) of the 20 subjects had ACL reconstruction only; while meniscus repair (medial, PL, n = 3; AO, n = 1; lateral, AO, n = 1) or meniscectomy (partial) (medial, PL, n = 2; AO, n = 1; lateral, PL, n =2) were performed on several subjects during ACL surgery as well. Of those subjects, one subject (PL) had both medial meniscus repair and lateral meniscectomy. Immediately following surgery, subjects were non-weight bearing with the knee immobilized in a leg brace (DonJoy, Vista, CA, USA) at 10º of knee flexion until their first post-surgery visit with the physician. The second biopsy (i.e., Post) was taken before resuming weight bearing. Days of non-weight bearing were recorded. Regardless of additional surgical procedures performed during ACL reconstruction, all subjects were allowed and able to initiate progressive weight bearing by 5 d post-surgery.

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)

Subjects were asked to refrain from using any NSAIDs, including aspirin, ibuprofen and naproxen sodium, throughout the duration of the study other than what was prescribed or recommended by the physician. As an alternative, subjects were

allowed to use acetaminophen. It is should be noted that the physician recommended one aspirin (325 mg) a day for the first 3-wks post-surgery.

Physical Therapy

Under the supervision of a physical therapist, pre- and post-surgery physical therapy was performed at one location (The Orthopedic Specialty Hospital, Murray, UT, USA). Pre-ACL reconstructive surgery therapy consisted of diminishing inflammation, swelling, pain, restoring knee range-of-motion, and voluntary muscle activation. Post-ACL surgery therapy consisted of re-establishing range-of-motion of the knee and voluntary contraction of the quadriceps. Functional movement exercises (i.e., gait training, etc.) were performed 1-3 wks post reconstructive surgery, and increased in intensity during the following weeks (i.e., 3-6 wks post reconstructive surgery). By approximately 8 wks post surgery, patients began impact and plyometric type activities. At 3-mo post-surgery, subjects were cleared to perform more intense activities if institutional criteria-based guidelines were achieved. All the subjects participating in this study performed a similar rehabilitation program (i.e., exercises or activities), but the intensity and volume were not rigidly constrained because we did not want to interfere or impede the subjects progression.

Analytical Procedures

Plasma Antioxidants

Plasma α-T and γ-T concentrations (μM) were measured using high-performance liquid chromatography (HPLC) with electrochemical detection, as described [283]. For measurements of ascorbic acid, immediately following the separation of heparinized plasma, an aliquot was acidified (1:1) with 5% metaphosphoric acid (MPA) containing 1 mM DTPA. The sample was then centrifuged (5 min, 15,000 x g; Mini Spin, Eppendorf, Hamburg), the supernatant removed, frozen in liquid nitrogen, and stored at -80ºC until analysis. AA plasma concentrations (μM) were measured using paired-ion reverse-phase HPLC coupled with electrochemical detection, as described [232].

Plasma F_2-Isoprostanes and Malondialdehyde (MDA)

Plasma 8-isoprostane prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and $PGF_{2\alpha}$ (pg/mL) were measured using liquid chromatography/mass spectrometry as described [355], in heparinized plasma samples that were kept frozen (-80 C) from the time of plasma isolation until analysis.

Plasma MDA (μM) was measured using HPLC with fluorimetric detection, as described [149, 398]. Briefly, 0.2 mL heparinized plasma or serial diluted standard (1, 1, 3, 3-tetraethoxy-propane, Sigma, St. Louis, MO, USA) was added to 0.1 mL 1N NaOH and incubated for 30 minutes at 60°C. Samples were cooled, then 1 mL 5% TCA (w:v, trichloroacetic acid) was added and the samples incubated 10 min on ice before centrifugation. Supernatant aliquots (0.5 mL) were mixed with 0.25 mL 0.6%

thiobarbituric acid (TBA; Sigma, St. Louis, MO, USA) and placed in boiling water for 30 min. Samples were then cooled on ice, extracted with 0.5 mL isobutyl alcohol. MDA-TBA was measured by HPLC with fluorescence detection (532 nm excitation, 553 nm emission) following separation using a reverse-phase C-18 column with an isocratic mobile phase containing 50% methanol and 50% 25 mM phosphate buffer at pH 6.5. Sample controls and standards were run with each batch.

Plasma Myeloperoxidase (MPO)

Myeloperoxidase (MPO; ng/mL) was measured in heparinized plasma samples at a dilution of 1:10 using an enzyme-linked immunoassay (BioCheck Inc., Forest City, CA, USA, BC-1129). Absorbance was measured spectrophotometrically at 450 nm (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA).

Plasma Cytokines and Serum High-Sensitivity CRP (hsCRP)

A multiplex microsphere-bead array was used to measure a number of circulating inflammatory cytokines (pg/mL) as previously described [26, 230]. Automated chemiluminescent immunoassay (IMMULITE/IMMULITE 1000 Automated Immunoassay, Diagnostic Products Corp., Los Angeles, CA) quantitatively measured serum hsCRP concentration (mg/L) [26].

Muscle Atrophy Assessed Histologically

Serially-sectioned, transversely cut muscle biopsies were viewed by a light microscopy for the purpose of measuring muscle fiber cross-sectional areas (FCSA; μm^2) and to assess atrophy (i.e., decrease in FCSA). Area measurements were performed using the Image J software from the National Institute of Health. A stage micrometer of several known distances (0.1, 0.25 and 0.5 μm) was used to calibrate and convert pixels to μm. Multiple fiber circumference measurements were carefully performed on each completely visible fiber. The technician was masked as to timing of the biopsy (Pre vs. Post) and to supplement (PL vs. AO).

Muscle Immunohistochemistry (IHC)

Serial-sections (4 μm) of the paraffin-blocks, cut using a microtome, were placed on a glass slide. Slides were heated in a microwave oven at 90º C for 10 minutes, cooled, then deparaffinized through two 5 minute changes in xylene, 3 rinses in absolute ethanol and six dips in 95% ethanol, and then rinsed in H_2O. Deparaffinized slides were placed in citrate buffer (0.1 M, pH 6.0) in a pressure cooker (pressurized and heated) for 50 minute for antigen retrieval. After the antigen retrieval, slides were rinsed with tap H_2O. Hydrogen peroxide (3%) was then applied to the entire slide for 5 minutes at room temperature. Slides were then rinsed with Tris Buffer (0.05 M, pH 7.6). The primary antibody (see below) was then applied for 10 minutes. After the overlay of the primary antibody, slides were rinsed in Tris Buffer and then overlayed with Dako ENV+ (a peroxidase-conjugated polymer with secondary antibody) for 10 minutes

(Dako Corporation, Carpinteria, CA, USA). Slides were subsequently rinsed in Tris Buffer. Diaminobenzidine (Dako, brown) was then applied for 5 minutes. Slides were counterstained with hematoxylin (Dako). All immunohistochemical staining was performed on a Dako Autostainer (Dako). Staining for a specific primary antibody was performed in one run on all biopsies with the Autostainer (Dako).

Positive and Negative Controls

Human lung (TNF-α and iNOS) and spleen (MPO) were used as positive controls. Negative control slides were prepared from each biopsy and performed by omitting the primary antibody. Positive and negative staining were confirmed by qualitative light microscopy (USB Microscopes, Saint Louis, MO, USA) and quantitatively with Clarient analysis (see below).

Primary Antibodies

The primary antibodies used were: polyclonal rabbit anti-human MPO (Dako Corporation, Carpinteria, CA, USA, A 0398, diluted 1:100 in Dako Antibody Diluent (S2022)), monoclonal mouse anti-human TNF-α (abcam, Cambridge, MA, USA, ab18696, diluted 1:100 in Dako Antibody Diluent), polyclonal rabbit anti-human iNOS (Thermoscientific, LabVision, Fremont, CA, USA, RB 9242 R7, pre-diluted), and a mouse monoclonal anti-human fast skeletal myosin (abcam, ab909, prediluted).

Image Analysis

After primary antibody staining (muscle biopsy tissues, including positive and negative controls), slides were scanned, measured and saved for later analysis using the Integrated optical density automated cellular imaging system application of Clarient (Medical Systems, Inc., San Juan Capistrano, CA, USA). From the saved image, five randomly selected areas from each slide were traced, measured for staining intensity, and averaged. Peripheral edges of the tissue where questionable slicing occurred from the biopsy needle were avoided during area measurements. The technician performing the area tracings was masked as to supplement (PL vs. AO) and biopsy timing (Pre- vs. Post-surgery).

Single Leg Strength Testing

All single leg strength testing was performed on a horizontal plyo-press (Athletic Republic, Fargo, ND, USA). Plyo-press output data was measured from a mounted force plate (Advanced Mechanical Technology Inc, Watertown, MA, USA) and displacement transducer (UniMeasure PA-50-NJC, Corvallis, OR, USA) output signals. The mounted force plate was used to measure ground reaction forces (N, newtons). The displacement transducer was used to measure the velocity (m/s) of the weight stack. All data was sampled at 200 Hz with a low-pass filter at 10 Hz. Prior to every testing session, the mounted force plate was zeroed and load (75% of subject's body mass) calibrated. Load calibration at 75% of body weight was selected because that was the load applied during the single leg power output tests (see below).

Single Leg Peak Isometric Force

SL isometric force measurements were performed on a horizontal plyo-press. Prior to every testing session, each subject performed a self-selected warm-up; which coincidently, consisted of a self-preferred pedal cadence (rpm) and external workload (watts) on a stationary bicycle for 5 to 10 minutes. Following warm-up, subjects were horizontally positioned on the plyo-press. The plyo-press sled was adjusted for each subject to align the knee and hip joint flexion angles to 90º with the abdominal, low back region secured and stabilized to the plyo-press sled with a harness. The plyo-press sled position was documented and reproduced to achieve the desired knee and hip joint flexion angles. Leg (i.e., hip and knee) extension-isometric contractions were elicited by overloading the weight stack resistance (> 2,260 Newtons, N). Three sub-maximal isometric contractions were performed on each leg (non-injured and injured) at ~50, 75 and 90% of the maximal effort. Following sub-maximal contractions, each subject performed three maximal single leg isometric contractions at each time point (i.e., Bsl and 3-mo). Leg selection at the start of each testing session was randomized and subsequently followed by an alternating leg sequence of contractions. Each isometric contraction was 3 s in duration and separated by at least 1 min of rest. Subjects were verbally instructed and encouraged to exert maximal force against the mounted force plate. The peak resultant isometric force applied against the mounted force platform were averaged over the three trials and expressed relative to body mass (N/kg). The peak isometric force was defined as the highest resultant force produced during the 3 s test for each leg separately.

Single Leg Peak Power Output

Single leg peak power output was measured on a horizontal plyo-press with the same securing procedures as described above (i.e., Single Leg Peak Isometric Force), and followed the single leg isometric contractions. From the extended position (full extension = 0º) on the plyo-press, subjects were instructed to perform repetitive single leg press (i.e., hip and knee flexion-extension) cycles as-fast-as possible while leaving their foot firmly placed on the mounted force plate. Order of leg (non-injured and injured) testing was randomized on each testing day. Each test was 20 s in duration with the weight-stack resistance set at 75% body weight. The product of the resultant force acquired from the force platform and weight stack velocity data obtained from the displacement transducer were used to calculate power output, and recorded and expressed relative to body mass (W/kg). Peak power output was defined as the highest power output produced during the 20 s test for each leg separately.

Statistical Analyses

To achieve a normal distribution of the data, log transformations were performed on the plasma α- and γ-T, AA, 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, and MDA concentrations, and a rank transformation was performed on MPO, cytokine concentration and cytokine ratio data. All transformed and non-transformed data were checked for normality and equal variance prior to and with our statistical analysis. To test our hypotheses, multiple linear regression analyses were performed to examine

the effect of treatment (AO vs. PL) and time (Bsl, Pre, 90-min, 72-h, 7-d and 3-mo) on plasma α- and γ-Ts, AA, 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, MDA, MPO, cytokines, the IL-6:IL-10 ratio, body mass, serum cholesterol and triglycerides and single leg strength measure outcomes.

With regard to the muscle biopsy data, to achieve normality prior to our statistical analysis, fiber cross-sectional areas (FCSAs) and all immunohistochemistry data were rank transformed. To test our hypothesis, multiple linear regression analyses were performed to (1) examine the effect of treatment (AO vs. PL) and time (Pre vs. Post) on muscle MPO, TNF-α and iNOS, and (2) the effect of treatment (AO vs. PL), time (Pre vs. Post-surgery) and myosin isoform (fast vs. non-fast myosin) on atrophy (i.e., change in FCSAs). Significant differences in the immunohistochemistry staining intensity change (i.e., Post minus Pre) were determined by a t-test.

Following a significant multiple linear regression, paired t-tests were performed to determine within group differences across time, and t-tests were performed to determine treatment group differences at various times points. A one-way ANOVA was performed on subject characteristics (i.e., height and age) and non-repeated measure outcomes (i.e., tourniquet pressure and duration, supplementation duration, and number of day's non-weight bearing post-surgery). A separate multiple linear regression analysis was performed to determine the effect of surgical procedures (ACL surgery only vs. ACL + meniscus repair vs. ACL + meniscectomy vs. ACL + meniscus + meniscectomy) and time on our various outcomes. Relationships between variables were examined with a Pearson Product Moment Linear Correlation analysis. All statistical analyses were performed with SysStat software (SigmaPlot 10.0, SigmaStat 3.5, Chicago, IL, USA). Statistical significance was set at a $P < 0.05$ (for correlation

coefficients (df = N – 2), N = 10, r ≥ 0.63; N = 20, r ≥ 0.44). Data presented as mean ± SEM, while the cytokine and hsCRP concentration data are presented as median (inter-quartile range).

Results

Subject Characteristics

General subject characteristic were similar between treatment groups; additional information has been published [26]. Specifically, subjects' age and height were similar upon study enrollment, tourniquet pressure and duration were not significantly different between treatment groups during surgery, and the non-weight bearing days post-surgery were comparable between groups as well [26]. Body mass, body mass index (BMI) and serum cholesterol and triglycerides were consistent between treatment groups upon enrollment and by 3-mo post-surgery (Table 4.1). Furthermore, supplementation duration prior to and following ACL surgery, and total duration of supplementation (pre plus post-surgery duration; PL, 102 ± 3; AO, 101 ± 2 d) were consistent between groups.

Finally, additional surgical procedures (i.e., meniscus repair and/or meniscectomy) performed during ACL reconstruction did not significantly influence our various outcomes measures.

Plasma Antioxidants

Circulating α- and γ-Ts were measured prior to (i.e., Bsl) and following supplementation (i.e., up to 3-mo) to evaluate the supplement influence on plasma concentrations; intermediate values have been previously reported, along with AA [26]. Upon enrollment, α- and γ-T were similar between groups. However, as anticipated,

circulating α-T remained increased and γ-T remained decreased following 3 mo AO supplementation (Figure 4.2 A and B; significant treatment x time interaction, $P < 0.001$). Compared to the PL group at 3-mo, AO supplementation increased α-T and decreased γ-T plasma concentrations.

Similar observations were apparent for both α- and γ-T when corrected for circulating lipids (Figure 4.2 C and D; significant ($P < 0.001$) treatment x time interaction). Specifically, α-T and γ-T per lipids were not significantly different between groups prior to supplementation. However, following supplementation (3-mo post-surgery), α-T per lipids increased, while γ-T per lipids decreased compared to those prior to surgery ($P < 0.05$) or to the corresponding PL values ($P < 0.05$).

Plasma AA concentrations were not significantly different between treatment groups at Bsl. However, there was a significant (treatment x time interaction, $P < 0.005$) increase in plasma AA following supplementation within the AO group and compared to the corresponding PL value (Figure 4.3).

Plasma F_2-isoprostanes and MDA

To assess oxidative stress, we measured two plasma markers of lipid peroxidation: malondialdehyde (MDA) and 8-isoprostane $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$). MDA concentrations did not change significantly over time or between treatment groups (Table 4.2).

With regard to plasma 8-iso-$PGF_{2\alpha}$ concentrations, which is one of the most reliable and valid markers of lipid peroxidation in humans [256], there were no significant effects of AO treatment, but there was an effect of time. Specifically,

following ACL surgery and tourniquet removal (i.e., 90-min), plasma 8-iso-$PGF_{2\alpha}$ concentrations were significantly elevated compared to all other time points (Figure 4.4 A). Bsl 8-iso $PGF_{2\alpha}$ concentrations were also significantly greater than that at 7-d and 3-mo. These findings suggest that an ACL injury and subsequent surgery induced lipid peroxidation, but that oral vitamin E and C supplementation was ineffective in decreasing this oxidative stress biomarker.

$PGF_{2\alpha}$, a marker of cyclooxygenase (COX)-2 activity [235], was measured simultaneously with 8-iso-$PGF_{2\alpha}$. $PGF_{2\alpha}$ concentrations were significantly higher before compared with after ACL surgery (Figure 4.4 B). Specifically, $PGF_{2\alpha}$ concentrations were greater at Bsl, Pre, 90-min or 3-mo compared with 72-h and 7-d; however, they were not significantly different between treatment groups. Taking into consideration the prescription of anti-inflammatory medication immediately following ACL surgery in these subjects [26], it is not surprising that $PGF_{2\alpha}$ was lower at 72-h and 7-d. However, in the same subjects and plasma samples, both IL-6 and hsCRP were elevated at 72-h and 7-d [26]; suggesting, that despite anti-inflammatory medication and the lowering of prostaglandins, surgery induced a systemic inflammatory response that persisted for several days.

Plasma Myeloperoxidase

Plasma myeloperoxidase (MPO), a key immunological defense enzyme [261], was measured to assess the prevalence of a potential source of inflammatory cell-derived oxidative stress. Plasma MPO concentrations were not significantly different across time ($P = 0.76$) or between groups ($P = 0.06$) (Table 4.2).

Plasma Cytokines and hsCRP

Among their numerous pleiotropic properties, inflammatory cytokines mediate muscle dysfunction [6, 89, 143, 205, 301, 383, 384]. We have previously reported the cytokine and hsCRP data from Bsl to 7-d post-surgery for the majority of the subjects described in the present report [26]. Here, we provide the cytokine and hsCRP concentrations measured at Bsl and 3-mo post-ACL surgery. hsCRP and the cytokines did not vary statistically significantly different across time (Bsl vs. 3-mo) or between supplements (PL vs. AO, Table 4.3)

Plasma IL-6 to IL-10 Ratios and Correlations

We previously found that AO supplementation ameliorated the increase in circulating IL-10 without significantly influencing the elevation of IL-6 following ACL reconstruction [26], but did not report the concentration ratio. Importantly, disturbances (i.e., increases or decreases) in the IL-6:IL-10 ratio may provide an important prognostic assessment of injury severity and post-surgery infection outcomes [318, 353, 354]. This ratio evaluates the balance between inflammatory cytokines (pro vs. anti), and may identify the prevalence of immuno-suppression. Based on the premise that under stressful conditions an exaggerated IL-10 response persists, which in turn depresses the IL-6:IL-10 ratio [318], we examined the IL-6:IL-10 ratios in our study subjects. Interestingly, the IL-6:IL-10 ratios displayed a significant ($P < 0.05$) treatment x time interaction (Figure 4.5 A). At 90-min post-surgery within the PL group, the IL-

6:IL-10 ratios were depressed compared to all other time points measured, and were depressed compared with the AO group 90-min value. These findings suggest that following ACL reconstruction there is a disruption to the pro- to anti- inflammatory cytokine balance characteristic of immuno-suppresion, which is stabilized with α-T and AA supplementation and suggestive of an improved recovery.

IL-6 and IL-10 have reciprocal regulatory effects on each other. IL-6 stimulates IL-10 production [69], while IL-10 inhibits IL-6 production [106, 251]. Furthermore, elevated circulating concentrations of IL-6 and IL-10 are correlated [125]; suggesting a coupling response between these cytokines. In agreement with this premise, we found a correlation between IL-6 and IL-10 at 90-min post-surgery in the PL group ($r = 0.72$, $P = 0.02$) (Figure 4.5 B). However, IL-6 and IL-10 were not significantly correlated in the AO group at 90-min ($r = 0.38$, $P = 0.28$). Thus, vitamin E and C supplementation appear to uncouple the association between a pro- and anti-inflammatory cytokine immediately following ACL surgery.

Muscle Morphology

Hemotoxylin & eosin (H&E) stains (Figure 4.6) were performed on every biopsy to assess general muscle morphology. Although no consistent pattern between treatment groups was qualitatively apparent, we observed centrally located nuclei and intra-fiber edema in several post-surgery biopsy specimens. Neither abnormality was apparent in the pre-surgery biopsy specimens. These findings suggest muscle regeneration and intra-fiber swelling within several days after surgery.

Muscle Fiber Atrophy

Muscle fiber circumference measurements were performed on each biopsy specimen to assess atrophy. Fiber cross-sectional areas (FCSA) of fast- or non-fast-myosin did not change significantly with time or within treatment groups (Pre- vs. Post-surgery: PL group, Figure 4.7 B and C, respectively; AO group, Figure 4.7 D and E, respectively). Interestingly, for both treatment groups there appeared to be a trend to decrease areas in non-fast myosin and increase areas in fast myosin from Pre- to Post-surgery. Therefore, we compared the ratio of fast- to non-fast-myosin fiber areas to identify if there was a disproportionate increase or decrease in fiber types across time and between groups. Surprisingly, there were no statistically significant differences between treatment groups (PL, 1.02 ± 0.08 and 1.23 ± 0.08; AO, 1.11 ± 0.09 and 1.19 ± 0.10 at Pre- and Post-surgery, respectively). It is noteworthy that our fiber type ratios (fast vs. non-fast myosin) are in close agreement with previous type I and II fiber type ratio data [22, 137].

Local Mediators of Muscle Atrophy

Muscle MPO (Figure 4.8), iNOS (Figure 4.9) and TNF-α (Figure 4.10) were measured to assess neutrophil infiltration, a potential source of inflammatory-derived oxidative stress, nitrative stress, and to identify local mediators of muscle atrophy. MPO, iNOS and TNF-α expression were not significantly different across time or between treatment groups. However, there was a trend for MPO and iNOS to decrease

in the PL group and increase in the AO group at Post- compared with Pre-surgery. There was also a trend for TNF-α staining to increase in the PL group at Post.

Based on these trends, we evaluated the staining intensity change for each individual (i.e., Post minus Pre). Interestingly, MPO ($P < 0.05$; Figure 4.8 I) and iNOS ($P < 0.05$; Figure 4.9 I) increased in the AO while they decreased in the PL group. These data suggest that vitamin E and C supplementation increased the infiltration of inflammatory cells and a potential source of nitric oxide (NO•) production following ACL surgery.

Single Leg Peak Isometric Force

Single leg peak isometric force measurements were conducted on both the non-injured and injured limbs for the purpose of investigating the influence of AO supplementation on the strength recovery of the injured limb following an ACL injury and surgery. With respect to the non-injured limb, peak isometric force did not significantly change with time or treatment.

In the PL group, the peak isometric force of the injured compared with the non-injured limb was ~24% less at Bsl ($P < 0.05$) and remained ~17% less at 3-mo post-surgery ($P < 0.05$). However, an improvement (~19%; $P < 0.05$) in the injured limb was noted when the 3-mo post-surgery peak isometric force was compared with that at Bsl (Figures 4.11 A and B).

In the AO group, the peak isometric force of the injured compared with the non-injured limb was ~26% less at Bsl ($P < 0.05$) and remained ~11% less at 3-mo ($P <$

0.05). However, comparing 3-mo post-surgery to Bsl, the peak isometric force of the injured limb did not significantly improve (~17%) (Figures 4.11 A and C).

Single Leg Peak Power Output

Peak power output measurements were also performed on each leg to further investigate the potential impact of AO supplementation on the recovery of strength, or power output, following ACL surgery (Figure 4.12). Differences in peak power output of the non-injured limb between and within treatment groups were not significant. The power output of the injured limb at 3-mo was significantly ($P < 0.05$) less than that of the non-injured limb at Bsl and 3-mo post-surgery in both groups.

Relationships Between Circulating Mediators of Muscle Dysfunction and Limb Strength

By 3-mo post-ACL surgery, the injured limb remained weaker than the non-injured limb, but improved in strength relative to Bsl measurements. Linear correlation analyses were performed to further investigate the association between plasma α-T or AA levels and the strength (peak isometric force) recovery of the injured limb following ACL reconstruction. Interestingly, plasma AA levels (PL and AO, $n = 20$) upon study enrollment (and prior to treatment) correlated ($r = 0.59$, $P = 0.006$) with the recovery of peak isometric force of the injured limb (Figure 4.13 A); suggesting that higher levels of circulating AA levels prior to surgery were associated with a greater recovery in peak

isometric force after ACL surgery. Similarly, one would expect lower oxidative stress levels to be associated with greater strength improvements. Consistent with this postulate, by 3-mo post-surgery, the greater the oxidative stress, as measured as plasma 8-iso-$PGF_{2\alpha}$, the poorer the recovery ($r = -0.76$; $P = 0.007$, Figure 4.13 B).

To examine whether this correlation existed separately in the PL and AO subjects, we performed a linear correlation on the post-surgery peak isometric forces and the recovery of peak isometric forces versus plasma 8-iso-$PGF_{2\alpha}$ levels in both treatment groups. In the AO group, the 3-mo plasma 8-iso-$PGF_{2\alpha}$ concentrations were inversely correlated with the recovery in peak isometric force (Figure 4.13 B; $r = -0.80$, $P = 0.005$) of the injured limb. In contrast, within the PL group, plasma 8-iso-$PGF_{2\alpha}$ concentrations were not correlated with strength recovery (Figure 4.13 B; $r = -0.14$, $P = 0.72$). These data indicate that relatively lower lipid peroxidation following AO supplementation was associated with greater strength and strength recovery following surgery.

Based on the premise that a pro-to-anti-inflammatory cytokine balance is optimal for ideal muscle function [89, 192], and that IL-6 [80, 191] and IL-10 [26, 198] are sensitive to oxidative stress and modulation by antioxidants, we examined the association between the IL-6:IL-10 concentration ratio with both limb strength and oxidative stress in both treatment groups. At 3-mo in the AO group, the IL-6:IL-10 ratio correlated with the peak isometric force of the injured limb (Figure 4.14 A, $r = 0.81$, $P = 0.005$) and inversely with plasma 8-iso-$PGF_{2\alpha}$ concentrations (Figure 4.14 B, $r = -0.77$, $P = 0.009$). Neither the recovery of force ($r = 0.10$, $P = 0.73$; data not shown) or 8-iso-$PGF_{2\alpha}$ ($r = -0.28$, $P = 0.43$; data not shown) correlated with the IL-6:IL-10 ratio in the PL group. Thus, as the IL-6 to IL-10 ratio increased the peak force production of the

injured limb also increased but only in the subjects that supplemented with vitamins E and C.

Discussion

Vitamin E and C supplementation increased mediators of muscle atrophy in the musculature of the surgically repaired limb several days after ACL surgery, and failed to improve strength recovery at 3 months. Immediately following surgery, prior vitamin E and C supplementation was ineffective in lowering oxidative stress but provided protection against immuno-suppression; with the latter response being indicative of an improved recovery following surgery [318, 353, 354]. Thus, contrary to our hypotheses, vitamin E and C supplementation does not appear to be beneficial in preventing or ameliorating muscle dysfunction following ACL surgery.

AO supplementation did not lower surgery induced oxidative stress. In agreement with previous ischemia-reperfusion data following ACL reconstructive surgery [319, 388], we observed an increase in circulating oxidative stress (Figure 4.4 A). Although the intravenous infusion of the antioxidant, N-acetyl-cysteine, effectively lowered oxidative stress (i.e., plasma MDA levels) following ACL reconstructive surgery [319], following vitamin E and C supplementation we were unable to reproduce similar results with a more reliable marker of oxidative stress (i.e., 8-iso-$PGF_{2\alpha}$). This was surprising because several human studies demonstrate that plasma or urine F_2-isoprostanes are decreased with combined vitamin E and C supplementation at various dosages (RRR-α-tocopherol or tocopheryl acetate, 150 to 400 IU/d; vitamin C, 500 to 1000 mg/d) and durations (4 week to 2 months) [86, 151, 232]. However, findings are inconsistent [87, 317], and with regard to the present study, may be accounted for by several factors.

It is plausible that our findings might be related to Bsl F_2-isoprostane levels that are not representative of a 'baseline' status, but rather reflective of a response to traumatic injury. Furthermore, the half-life and elimination of plasma 8-iso-$PGF_{2\alpha}$ is extremely short; in the order of minutes [32]. Therefore, it is possible that we missed a potential treatment effect based on our temporally designed blood draw protocol. Another explanation for our inability to identify a treatment effect on F_2-isoprostanes may be due to our daily dose of vitamin E (400 IU); which, arguably, was low to elicit a treatment effect [308]. However, under different conditions, the Traber group previously demonstrated that a similar dose and form of α-T (combined with AA) ameliorated the F_2-isoprostane increase following a ultra-marathon in healthy adults [232]. Finally, anesthesia and inflammatory medication likely affected our oxidative stress outcomes immediately following surgery. For example, propofol displays antioxidant properties [4, 5, 164]. However, medication (pain and inflammatory) and anesthesia were consistent between subjects; and despite the reduction of a marker of COX-2 activity at 72-h and 7-d (Figure 4.4 B), we previously reported at those same time points and in the same subjects that there is a profound systemic inflammatory response [26]. Nonetheless, our results support the premise of an increase in oxidative stress following ACL surgery [319, 388]. When taken collectively with our previous results [26], they indicate a distinct catabolic state (i.e., increase in oxidative stress and pro-inflammatory cytokines) following ACL surgery; which theoretically, may contribute to muscle atrophy following ACL surgery.

AO supplementation uncoupled the correlation between IL-6 and IL-10. Another novel finding of the present study is that it identifies the importance of integrating a multifaceted data analysis approach when investigating and interpreting the balance

between reciprocally regulated and highly variable cytokines that possess pleiotropic and redundant immunological and physiological properties. Although we previously reported the immuno-modulatory properties of α-T and AA following ACL surgery [26], we extend this knowledge in the present study by demonstrating the significance of a stable IL-10 response following AO supplementation relative to IL-6.

Among the abundant cytokine feedback loops, it is well recognized that IL-6 stimulates IL-10 production [69] and that an elevation in IL-10 inhibits the production of IL-6 [106, 251]. Furthermore, higher circulating IL-6 concentrations are correlated with higher circulating IL-10 in patients suffering from community acquired pneumonia [125]. IL-10 levels in humans also correlate with the severity of sepsis [126], systemic inflammatory reaction [371] and systemic lupus erythematosus [53]. In the present study, we found a correlation (Figure 4.5 B) between IL-6 and IL-10 at 90-min post-surgery. Remarkably, higher IL-6 levels were not associated with higher IL-10 levels following AO supplementation (Figure 4.5 B). This finding suggests a dissociation between a pro- and anti-inflammatory cytokine immediately following ACL surgery with prior AO supplementation. Although IL-10 concentrations are inconsistently correlated with trauma severity [354], changes in the IL-6:IL-10 concentration ratios have developed into a clinical parameter of potential prognostic promise [318, 353].

AO supplementation provided protection against immuno-suppression. In the present study, we found that AO supplementation prevented the decrease in the IL-6:IL-10 ratio at 90-min post-ACL surgery observed in the PL group (Figure 4.5 A). Changes in this ratio have been associated with adverse outcomes. For example, lower IL-6:IL-10 ratios were associated with an increased rate of infectious complications following surgery in chronic alcoholics [318]. However, following chest

and abdominal trauma, the IL-6:IL-10 ratio positively correlated with injury severity [354]. Moreover, increases in the IL-6:IL-10 ratio predict a poor outcome in patients with systemic inflammatory response syndrome [353]. These findings collectively indicate that either an increase or decrease in the IL-6:IL-10 ratio predict a poor clinical outcome. Although additional studies are warranted to examine the prognostic potential of alterations in the IL-6:IL-10, we found that the AO supplementation beneficially stabilized the IL-6:IL-10 ratio immediately following ACL surgery. Interestingly, both organ failure and intensive care unit stay were reduced in critically ill patients that received α-T orally and AA intravenously [259], but that study did not report IL-6:IL-10 ratios.

Post-surgery atrophy, or lack thereof. Surprisingly, our data indicates an absence of significant atrophy several days post-ACL surgery. Literature pertaining to muscle atrophy prior to ACL surgery indicates that type I or non-fast muscle fibers are distinctly smaller prior to surgery [96], while others have reported inconsistent results [223]. Furthermore, type II or fast myosin fiber areas are reported to be smaller at 6 wks to 1 yr post-ACL surgery [22, 222], while other have reported that the areas of type II fibers increase and type I fibers decrease following 5 wks of limb immobilization post-ACL surgery [137]. As would be anticipated based on these inconsistent results, type II-to-type I fiber ratio discrepancies are also prevalent following ACL surgery [22, 137]. Thus, the decrease in non-fast myosin (type I) areas, and the increase in fast myosin (type II) areas and the type II-to-type I fiber ratio trends following surgery are not overly surprising. However, it is coincidently provoking that fast myosin fibers displayed a swelling or necrotic response, and that the atrophy or the inability to recruit type II fibers contribute to muscle dysfunction following an ACL injury and surgery [240, 340].

Nonetheless, we did not find a significant atrophy response by 5-d post-surgery; moreover, there were no significant differences in fiber cross-sectional areas between treatment groups.

It is possible that a rapid atrophy response occurred after injury but prior to ACL surgery; thereby masking our atrophy measurements that were performed on biopsies obtained immediately before and several days after surgery. Another possible explanation for our inability to identify a significant atrophy response may be that a local inflammatory response either prior to or immediately after surgery (or both) contributed to fiber swelling and confounded our fiber size and atrophy measurements. Finally, it is plausible that modern advances in medicine and surgery have improved the morphological recovery compared to earlier biopsy studies that examined atrophy following ACL surgery.

Local mediators of atrophy increased in the non-atrophied muscle following AO supplementation. A major finding of the present study was that vitamin E and C supplementation increased muscular iNOS several days after ACL surgery in the absence of overt muscle atrophy. Among other regulatory mechanisms, IL-6 increases iNOS protein expression in muscle (myoblast) cell culture [116], while higher levels of IL-10 are associated with the decreased protein expression of iNOS in human atherosclerotic plaques [225]. Thus, our findings support the premise that following AO supplementation an elevated pro-inflammatory resulted from the blunted anti-inflammatory cytokine response, and thereby augmented the infiltration of inflammatory cells (based on MPO measurements and the expression of iNOS). The previously reported detrimental influence of iNOS on peripheral skeletal muscle function in patients suffering from chronic heart failure [140, 305], and its ability to mediate

inflammatory cell infiltration and muscle injury (i.e., edema, atrophy, etc.) following ischemia-reperfusion [291, 400], suggests, in theory, a potential harmful effect of vitamin E and C. Conversely, is it possible that an increased iNOS expression is beneficial?

Increased iNOS may have provided protection against tissue injury following ischemia-reperfusion by elevating nitric oxide (NO•) production, which subsequently scavenged various reactive oxygen species and minimized pro-inflammatory processes [278]. Evidence supporting this hypothesis is provided by studies in experimental animals (i.e., mice, rats, rabbits) that demonstrated an increase of iNOS expression following ischemia-reperfusion pre-conditioning resulted in a beneficial response in cardiac muscle (i.e., decreased of infarct size or contractility impairment) [63, 134, 204, 352, 395]. Whether the change in skeletal muscle iNOS following ACL surgery and AO supplementation was beneficial or detrimental awaits future studies, but it does identify an interesting regulatory influence of vitamins E and C on a local mediator of muscle dysfunction in humans following ACL reconstructive surgery.

Antioxidant supplementation did not improve strength recovery following ACL surgery. Upon study enrollment and by 3-mo post-surgery, the injured limb was significantly weaker than that of the non-injured limb. Interestingly, the injured limb within the PL, but not the AO group, displayed a significant increase from Bsl to 3-mo (Figure 4.11). Based on this finding, it is possible that AO supplementation was detrimental on strength recovery following ACL reconstruction. However, several provocative relationships between circulating mediators of muscle dysfunction and the strength recovery of the injured limb were observed within the AO but not the PL group. For example, greater strength and strength recovery of the injured limb following

surgery were associated with a higher IL-6:IL-10 ratio (Figure 4.14 A) and lower circulating F_2-isoprostane (Figure 4.13 B) levels, respectively (see below).

AO supplementation inversely associated oxidative stress and strength gains following surgery. Oxidative stress is a pivotal mediator of skeletal muscle dysfunction. As recently reviewed [286], experimental studies ranging from in vitro muscle preparations to in vivo human studies identify the detrimental influence of oxidative or nitrative stress on muscle function [10, 48, 55, 146, 241, 249, 250, 303, 348, 364]. Although a discernible difference in peak isometric force following ACL surgery was not observed between treatment groups, our finding that lower oxidative stress levels are associated with greater peak force and force gains (i.e., recovery) of the injured limb following surgery is consistent with this premise.

A higher pro- to anti-inflammatory ratio was associated with greater strength following surgery and AO supplementation. Similar to oxidative stress, increased concentrations of inflammatory cytokines (i.e., TNF-α, IL-6, etc.) are associated with increased muscle weakness in humans [62, 119, 257, 320, 373], while experimental animal studies clearly establish pro-inflammatory cytokines as causative determinants of weakness [6, 89, 205, 301, 383, 384]. However, given the reported importance of a balance between pro- and anti-inflammatory cytokines on muscle function [89, 192], we investigated the IL-6:IL-10 ratio relative to post-surgery strength.

By 3-mo post-ACL surgery, the IL-6:IL-10 ratio correlated with the peak force production of the injured limb (Figure 4.13 A) in the AO, but not the PL, group. In IL-10 knockout mice, Kryzston et al. [192] recently demonstrated that IL-10 deficiency exacerbated the pro-inflammatory cytokine (i.e., IL-1β, IL-6, etc.) increase and the subsequent muscle weakness response to lipopolysaccharide stimulation; confirming

the importance of a pro-to-anti-inflammatory cytokine balance on muscle function [89]. The present report corroborates the notion that a balance between pro- and anti-inflammatory cytokines is needed for optimizing muscle function, but extends this knowledge by translating this association into a human population suffering from limb weakness following orthopedic surgery and AO supplementation.

Circulating AA prior to supplementation and surgery correlated with strength recovery following surgery. A novel finding of the present investigation was that circulating AA levels prior to ACL surgery correlated with the strength recovery of the injured limb following surgery. This finding suggests that subjects with greater plasma vitamin C levels following an ACL injury but prior to surgery possessed a greater strength recovery following reconstruction. A similar observation between AA and limb weakness has been shown with aging; specifically, dietary intake of AA (and plasma α-T concentrations) correlated with limb strength in elderly [61]. With advancing age there is a significant increase in oxidative stress [29, 101, 124, 242, 268] which, among other factors (i.e., IL-6, TNF-α, etc.) [49, 130, 373], may mediate age-associated weakness [150]. In theory, increasing the dietary intake of AA increases plasma AA, reduces oxidative stress and restores strength capacity. Additionally, AA possesses other physiological functions as well.

AA acts as a co-substrate for hydroxylase enzymes involved in the biosynthesis of collagen [201]. Previously, human ACL cells incubated in vitro with ascorbate-2-phosphate, a long-acting derivative of AA, enhanced collagen synthesis [103]. Similarly, in smooth muscle cells, AA stimulated collagen (type I) synthesis [293]. Thus, it is possible that greater AA concentrations increased collagen synthesis, accelerated the strength development of the new graft and minimized the post-surgery strength

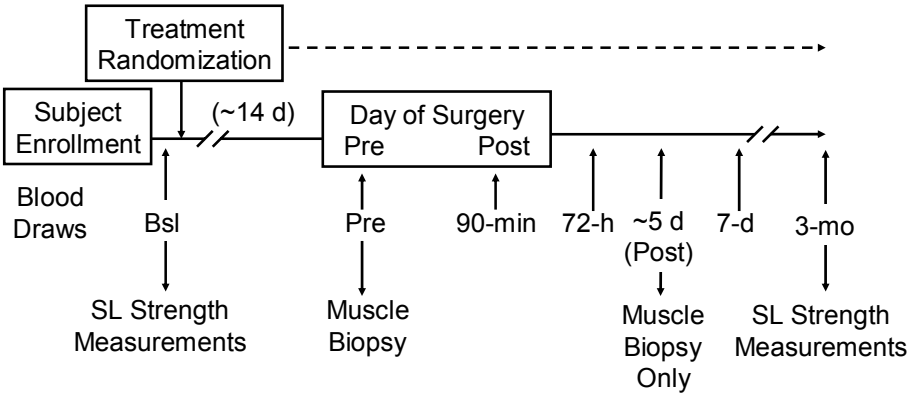
(muscle and graft) impairments. It is also plausible that AA enhanced collagen synthesis in skeletal muscle and maintained the structural integrity interface between the muscle fiber and connective tissue. Nevertheless, greater AA levels in the circulation following an ACL injury and prior to surgery appeared to be advantageous on strength recovery post-reconstruction.

AO supplementation: beneficial or detrimental on muscle following an ACL injury and surgery, and a possible nutrient-drug interaction? The findings from the present study provide evidence that vitamin E and C supplementation was ineffective and potentially exacerbated muscle dysfunction following ACL surgery. With regard's to the collective sum and interpretative clarity of our results, it is important to appreciate that our subjects received typical outpatient treatment (or care) with regard to prescribed anti-inflammatory medication and recommended aspirin consumption; which may have affected our outcomes and likely contributed to a nutrient-drug interaction. For instance, in addition to its anti-thrombotic effects, aspirin minimizes oxidative stress-induced toxicity by increasing the expression and catalytic activity (i.e., increase in bilirubin formation) of the antioxidant enzyme, heme oxygenase-1 (HO-1), in a NO•-dependent and cyclooxygenase-independent manner in human endothelial cells [133]. This finding raises the question, did combined AO and aspirin treatment facilitate the increase in inflammatory cells that express iNOS and presumably increase NO• production protect against muscle dysfunction? Although additional research addressing this postulate is warranted, the findings from the present study suggest that AO supplementation was ineffective or detrimental on muscle following an ACL injury and surgery.

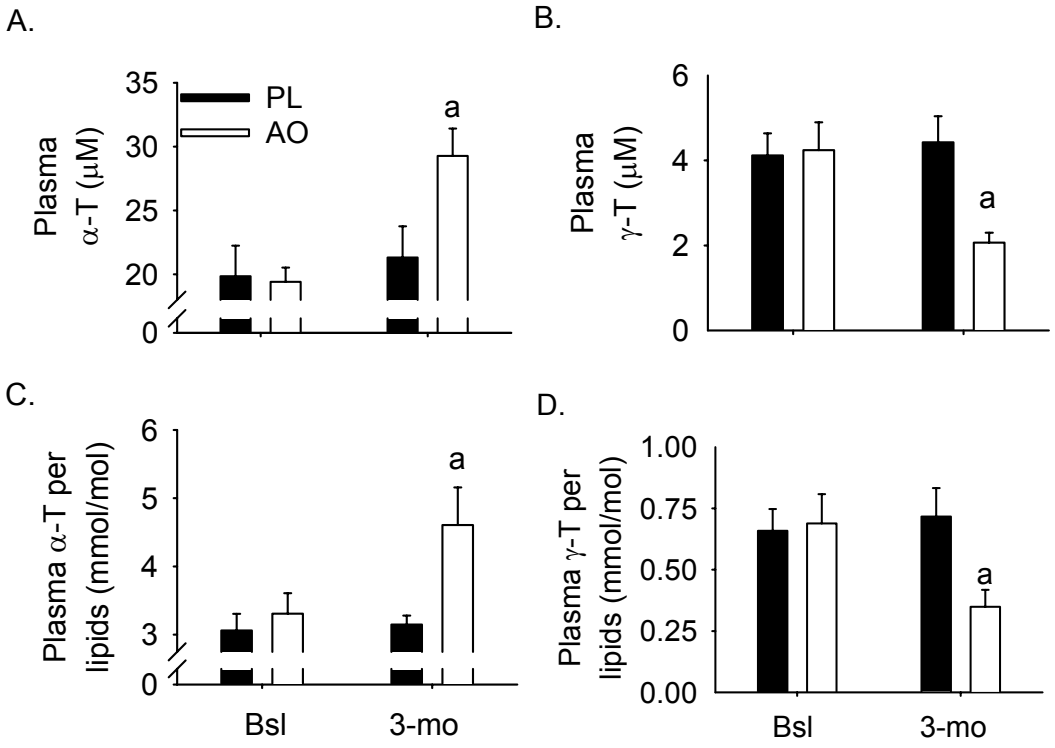
Summary:

We provide novel data showing that vitamin E and C supplementation increased mediators of muscle atrophy in the musculature of the surgically repaired limb several days after ACL surgery, and failed to improve strength recovery at 3 months. Immediately following surgery, prior vitamin E and C supplementation was ineffective in lowering oxidative stress but provided protection against immuno-suppression; with the latter response being indicative of an improved recovery following surgery [318, 353, 354]. Thus, contrary to our hypotheses, vitamin E and C supplementation does not appear to be beneficial in preventing or ameliorating muscle dysfunction following ACL surgery.

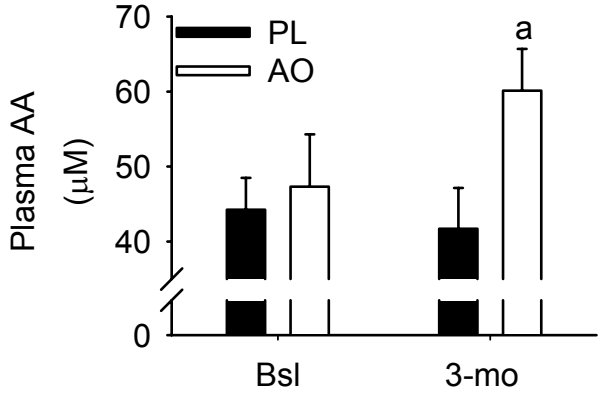
Figures



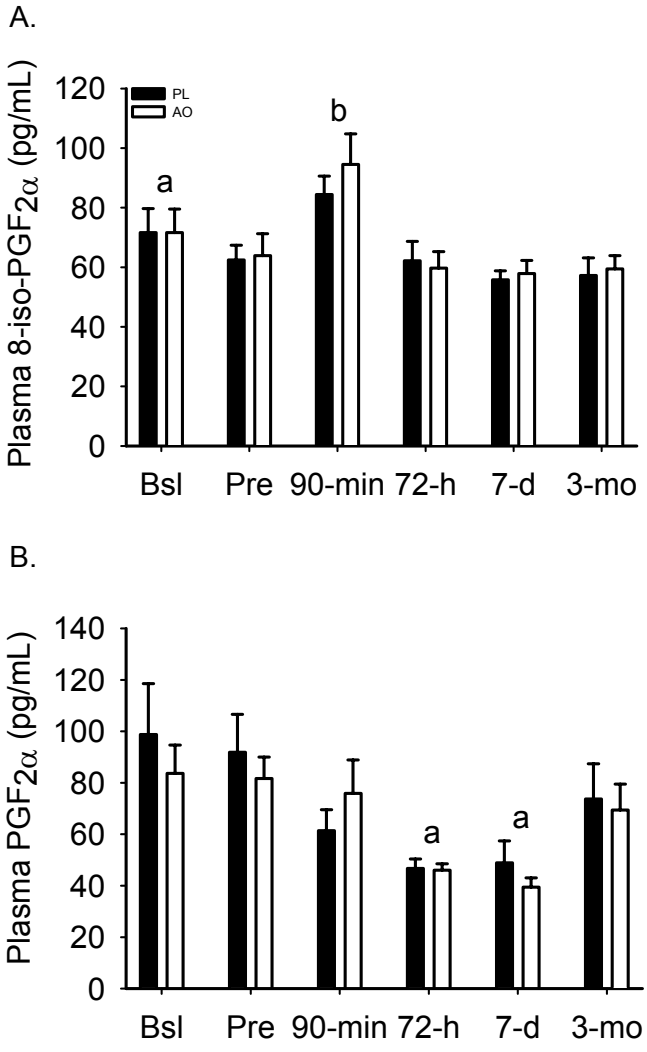
4.1. Study Protocol. Bsl, baseline. SL, single-leg.



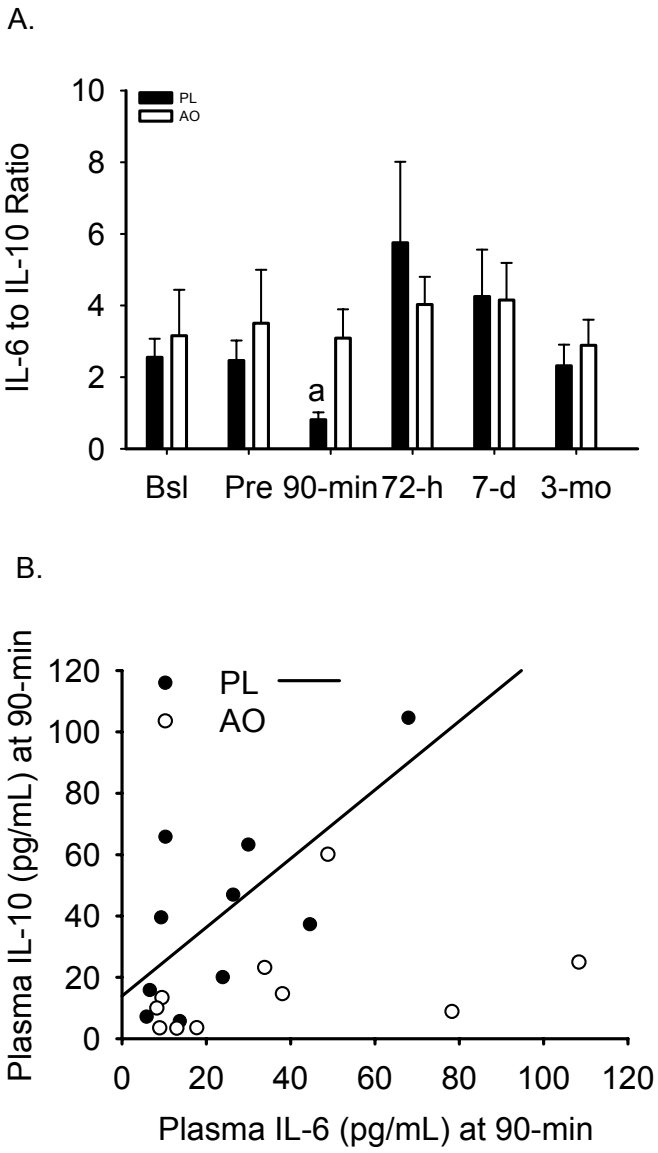
4.2. Plasma α-Tocopherol (α-T) and γ-Tocopherol (γ-T) Prior To and 3-mo Post-ACL Surgery in Both Treatment Groups (PL vs. AO). There was a significant treatment x time interaction ($P < 0.001$) for both α-T (A, μM; C; relative to lipids (cholesterol + triglycerides; mmol/mol)) and γ-T (B, μM; D, relative to lipids (cholesterol + triglycerides; mmol/mol)). AO supplementation significantly ($^{a}P < 0.05$) increased α-T and decreased γ-T concentrations and relative to lipids compared to Bsl and PL 3-mo. Data presented as mean ± SEM.



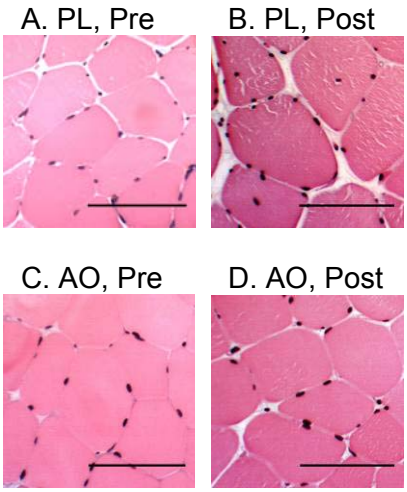
4.3. Plasma Ascorbic Acid (AA) Prior To and 3-mo Post-ACL Surgery in Both Treatment Groups (PL vs. AO). There was a significant treatment x time interaction ($P < 0.005$). AO supplementation significantly ($^{a}P < 0.05$) increased AA concentrations compared to Bsl and PL 3-mo. Data presented as mean ± SEM.



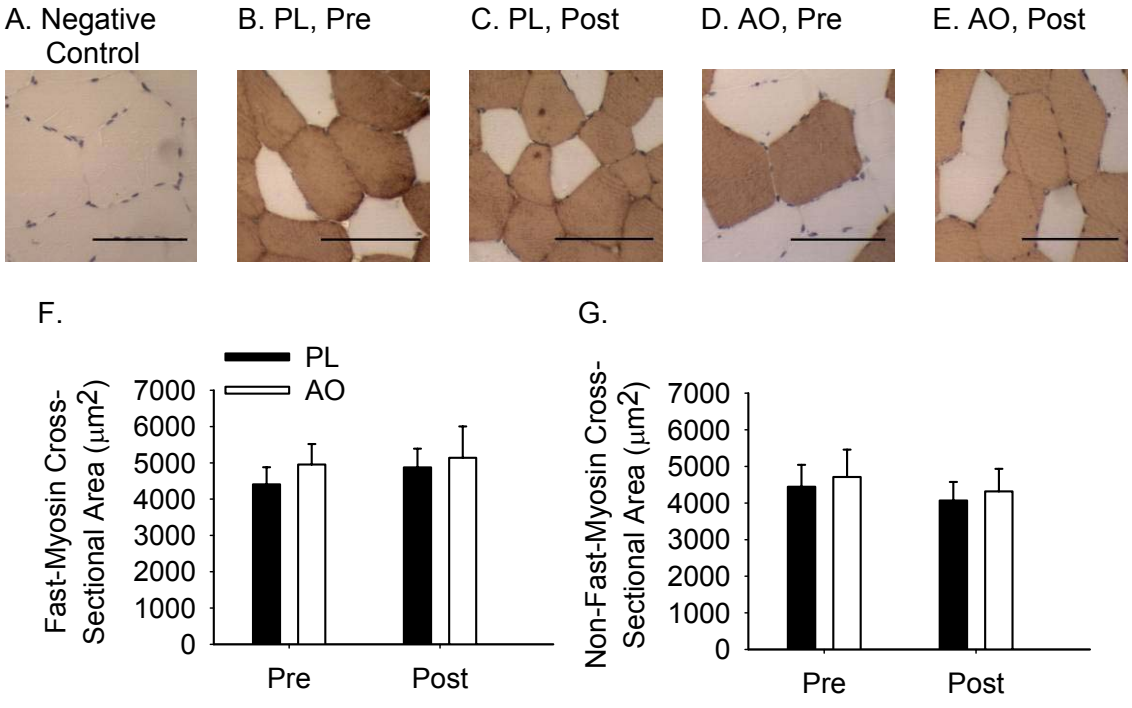
4.4. Plasma 8-Isoprostane Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) (A) and $PGF_{2\alpha}$ (B). (A) Upon enrollment and prior to supplementation and surgery (i.e., Bsl), plasma 8-iso-$PGF_{2\alpha}$ (pg/mL) concentrations were significantly ($^{a}P < 0.05$) elevated compared to that at 7-d and 3-mo. At 90-min, plasma 8-iso-$PGF_{2\alpha}$ concentrations were significantly ($^{b}P < 0.05$) elevated compared to all other time points. There were no significant differences in plasma 8-iso-$PGF_{2\alpha}$ levels between treatment groups. (B) Plasma $PGF_{2\alpha}$ (pg/mL) concentrations were significantly ($^{a}P < 0.05$) lower at 72-h and 7-d compared to Bsl, Pre, 90-min and 3-mo. $PGF_{2\alpha}$ levels were not significantly different between 72-h and 7-d, and were unaffected by treatment. Data presented as mean ± SEM.



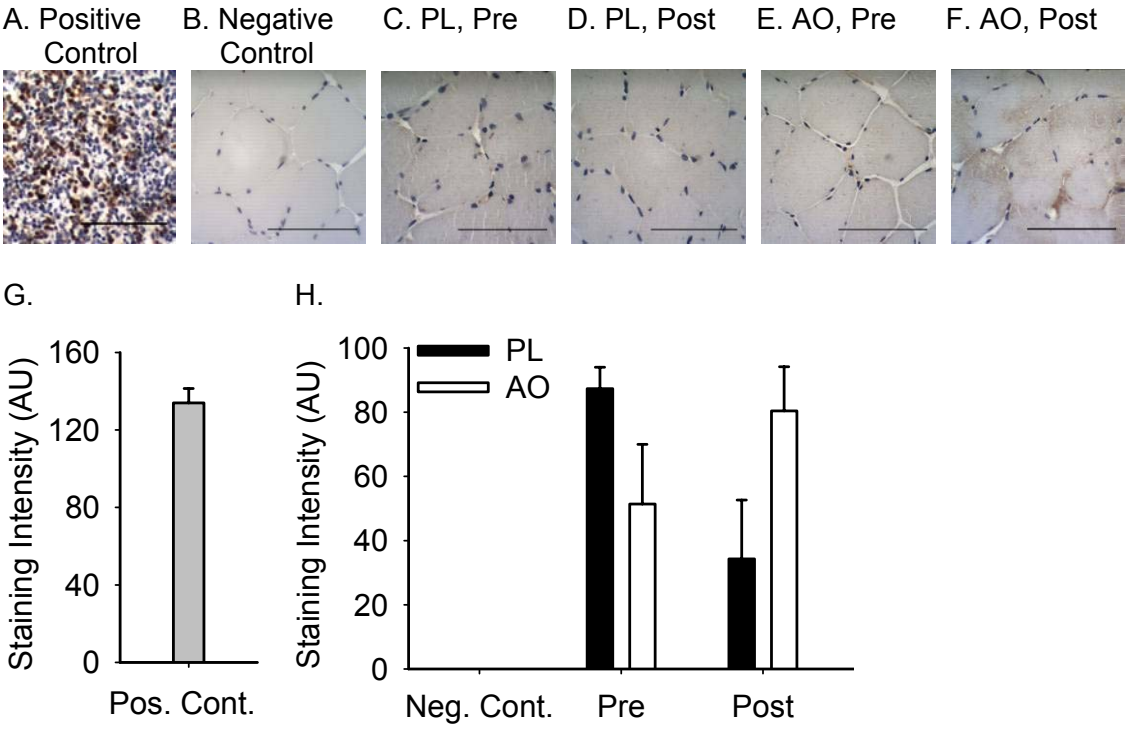
4.5. Relationship of Interleukin (IL)-6 and IL-10 in ACL Injury Patients With PL or AO Supplementation. (A) IL-6:IL-10 concentration ratios displayed a significant treatment x time interaction ($P < 0.05$). At 90-min post-surgery, there was a significant ($^{a}P < 0.05$) depression in the IL-6:IL-10 ratio compared to that at Bsl, Pre, 72-h, 7-d and 3-mo in the PL group. Moreover, AO supplementation provided protection against this depression. Data presented as mean ± SEM. (B) Plasma concentrations of IL-6 and IL-10 at 90-min post-surgery correlated in the PL ($r = 0.72$, $P = 0.02$, —) but not in the AO ($r = 0.38$, $P = 0.28$) group.



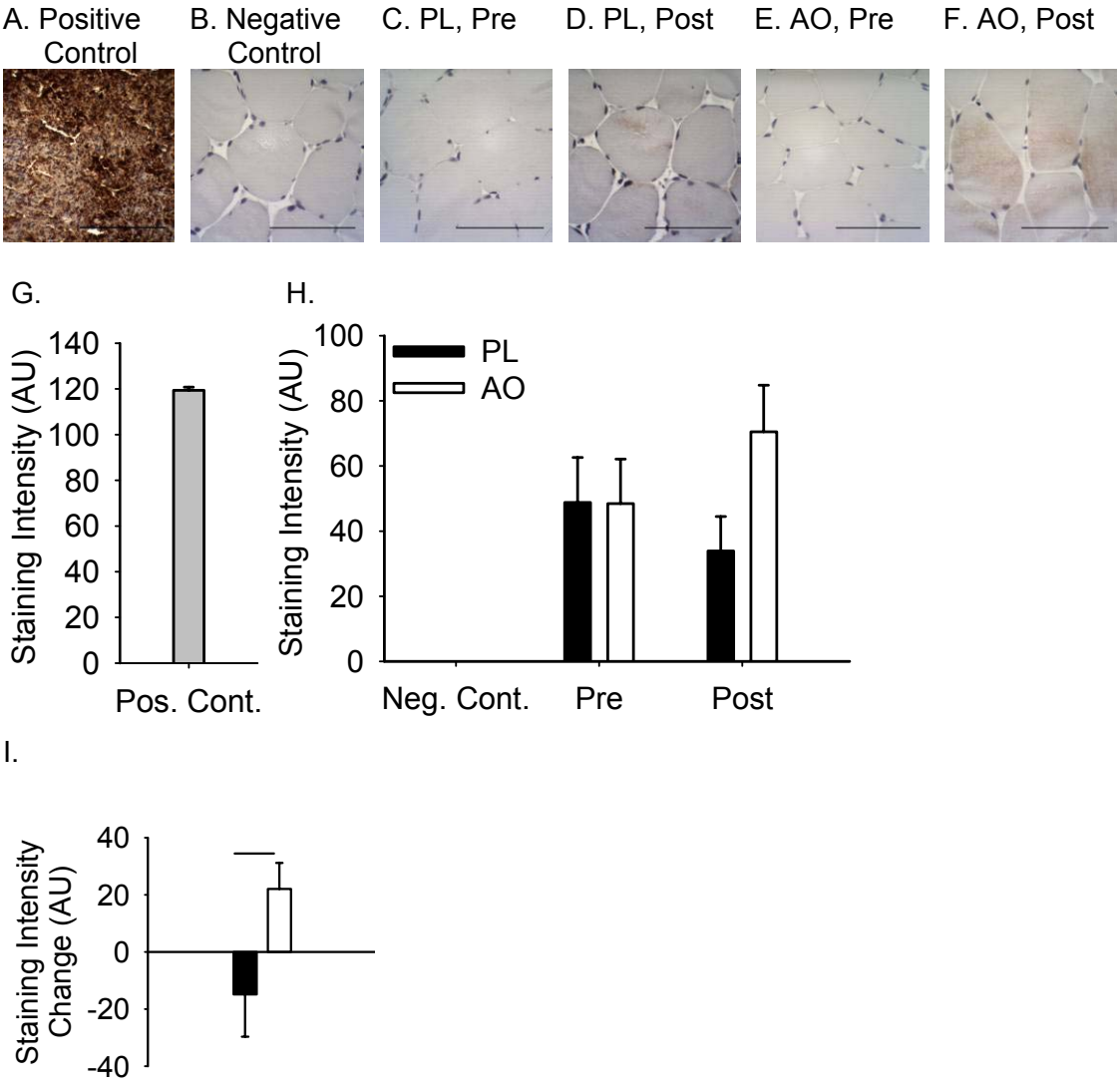
4.6. Hemotoxylin and Eosin (H&E) Stains. Examples of an H&E stain for a PL (A, Pre; B, Post) and AO (C, Pre; D; Post) subject. Images originally captured at a magnification of 200X. Scale bar equals 100 μm.



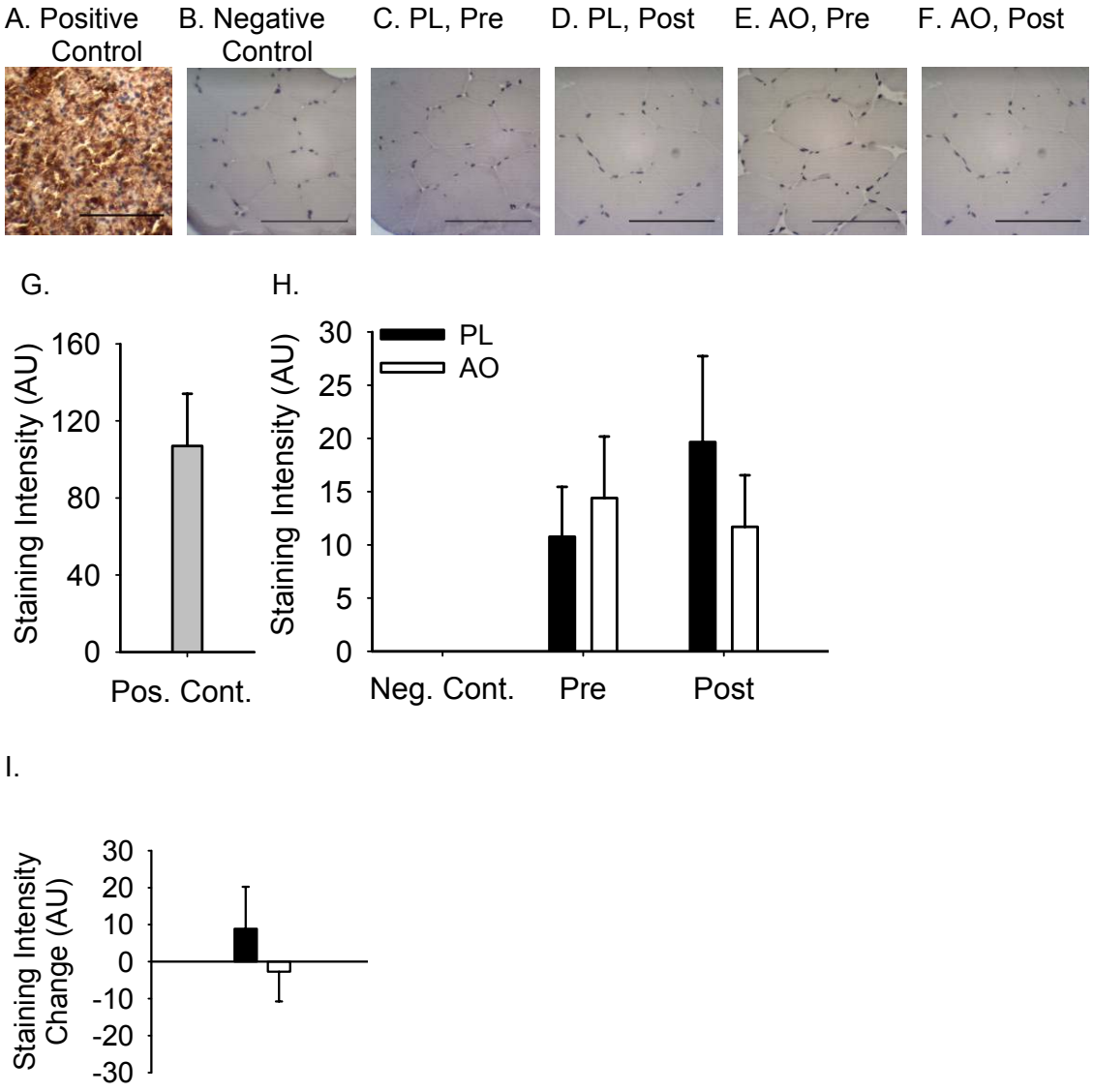
4.7. Fast- and Non-Fast-Twitch Myosin Staining Images and Descriptive Statistics. (A) Negative control; same biopsy as seen in 'D'. Example of a PL subjects' Pre (B) and Post (C) surgery biopsy specimen. Example of a AO subjects' Pre (D) and Post (E) surgery biopsy specimen. Descriptive statistics for fast (F) and non-fast (G) myosin fiber cross-sectional areas (μm^2). Differences between and within groups were non-significant. Data presented as mean ± SEM. Images originally captured at a magnification of 200X. Scale bar equals 100 μm.



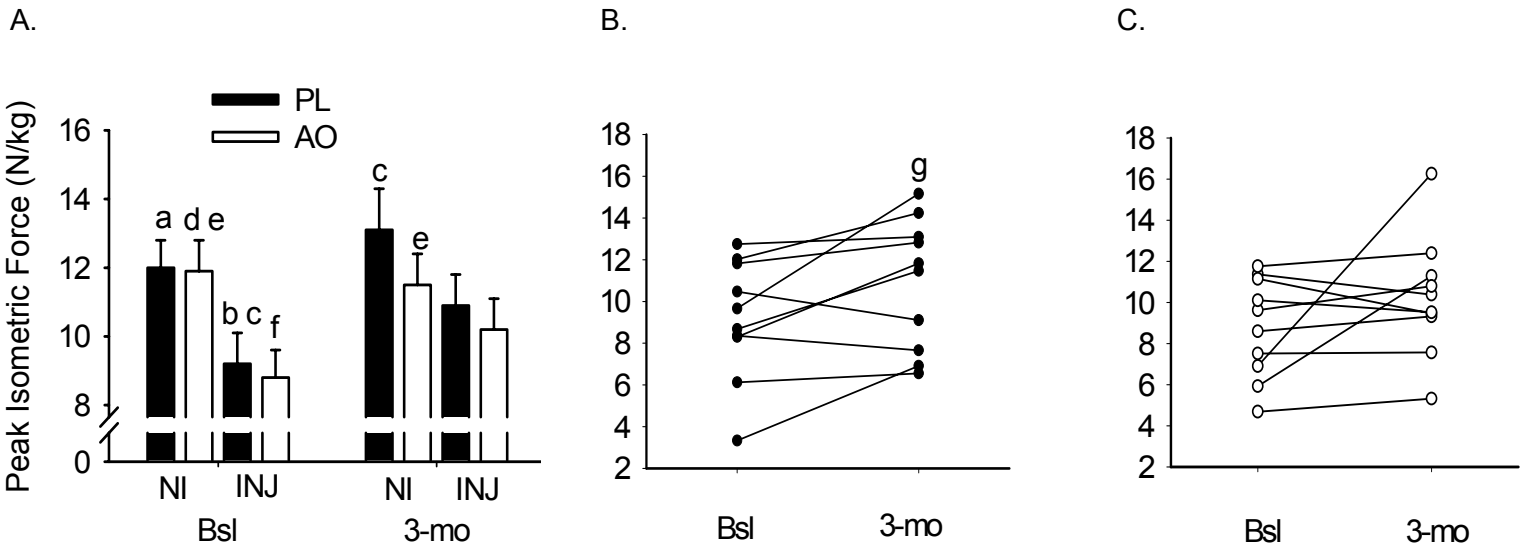
4.8. Example of The Immunohistochemistry Staining and Group Descriptive Statistics for Myeloperoxidase (MPO). (A) Human lung; positive control. (B) Negative control; same biopsy sample as seen in 'E'. Example of a PL subjects' Pre (C) and Post (D) surgery biopsy specimens. Example of a AO subjects' Pre (E) and Post (F) surgery biopsy specimens. Positive control (Pos. Cont.; human lung) (G), and negative control (Neg. Cont.), Pre and Post (H) group descriptive statistics. (I) The staining intensity change from Pre (bar, $P < 0.05$). Data presented as mean ± SEM. Images originally captured at a magnification of 400X. Scale bar equals 100 μm.



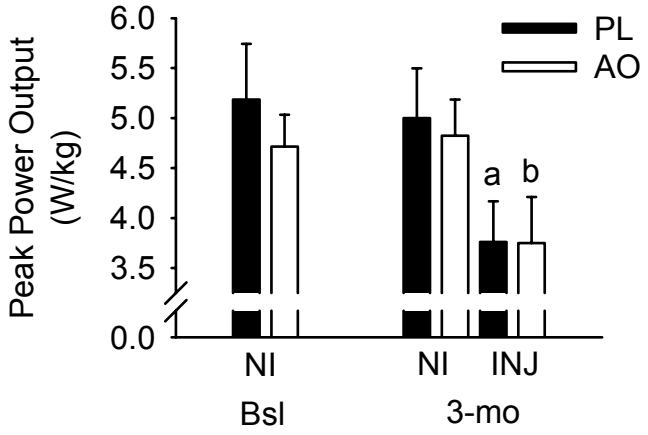
4.9. Example of The Immunohistochemistry Staining and Group Descriptive Statistics for Inducible Nitric Oxide Synthase (iNOS). (A) Human lung; positive control. (B) Negative control; same biopsy sample as seen in 'D'. Example of a PL subjects' Pre (C) and Post (D) surgery biopsy specimens. Example of a AO subjects' Pre (E) and Post (F) surgery biopsy specimens. Positive control (Pos. Cont.; human lung) (G), and negative control (Neg. Cont.), Pre and Post (H) group descriptive statistics. (I) The staining intensity change from Pre (bar, $P < 0.05$). Data presented as mean ± SEM. Images originally captured at a magnification of 400X. Scale bar equals 100 μm.



4.10. Example of The Immunohistochemistry Staining and Goup Descriptive Statistics for Tumor Necrosis Factor-α (TNF-α). (A) Human spleen; positive control. (B) Negative control; same biopsy sample as seen in 'D'. Example of a PL subjects' Pre (C) and Post (D) surgery biopsy specimens. Example of a AO subjects' Pre (E) and Post (F) surgery biopsy specimens. Positive control (human spleen) (G), and negative control, Pre and Post (H) group descriptive statistics. (I) Staining intensity change from Pre. Data presented as mean ± SEM. Images originally captured at a magnification of 400X. Scale bar equals 100 μm.

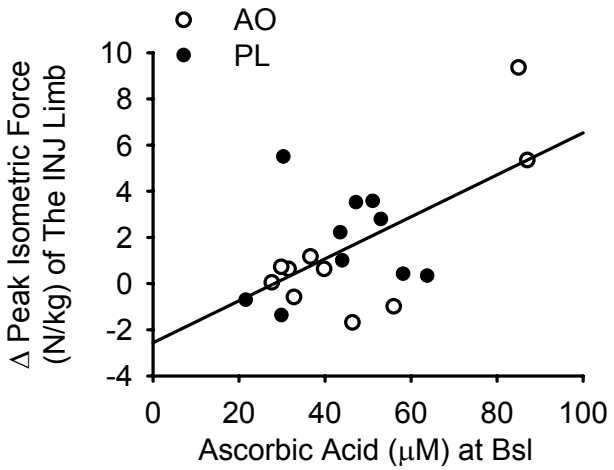


4.11. Single-Leg (SL) Peak Isometric Force (N/kg) of The Non-Injured (NI) and Injured (INJ) Limbs at Bsl and 3-mo Post-ACL Surgery in Both Treatment Groups (A. AO vs. PL), and The SL Peak Isometric Force of The INJ for Each Subject (B, PL Subjects; C, AO Subjects). (A) [a]Significantly ($P < 0.05$) different from PL INJ at Bsl. [b]Significantly different from PL NI at 3-mo. [c]Significantly different from PL INJ at 3-mo. [d]Significantly different from AO INJ at Bsl. [e]Significantly different from AO INJ at 3-mo. [f]Significantly different from AO NI at 3-mo. No significant differences were found between treatment groups. Data presented as mean ± SEM. Peak isometric force (N/kg) of the INJ limb at Bsl and 3-mo for each subject in both the PL (B) and AO (C) treatment groups. [g]Significantly ($P < 0.05$) different from Bsl.

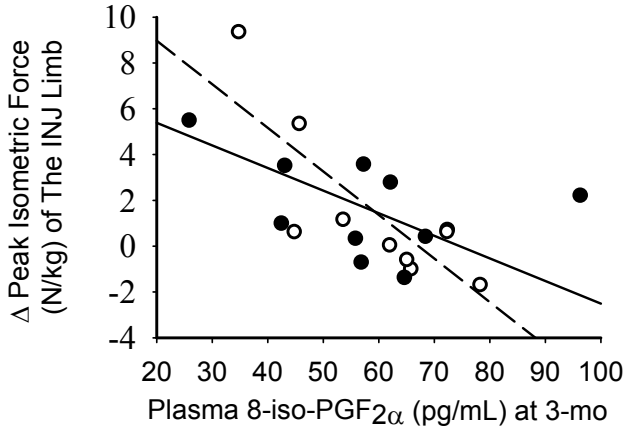


4.12. Single-Leg (SL) Peak Power Output (W/kg) of The Non-Injured (NI) and Injured (INJ) Limb at Bsl and 3-mo Post-ACL Surgery in Both Treatment Groups (PL vs. AO). [a]Significantly ($P < 0.05$) different from PL NI at Bsl and 3-mo. [b]Significantly ($P < 0.05$) different from AO NI at Bsl and 3-mo. Data presented as mean ± SEM.

A.



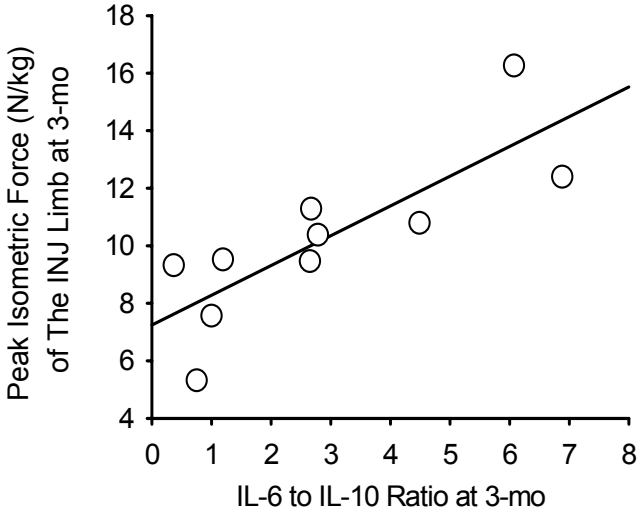
B.



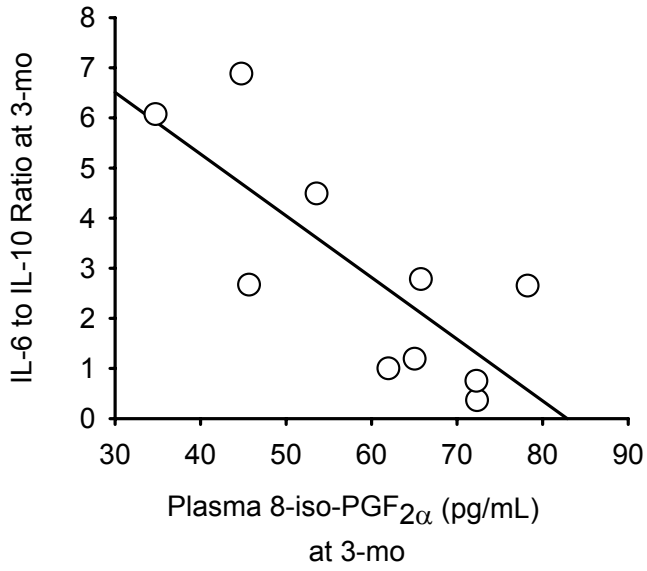
4.13. Correlation Between Plasma Ascorbic Acid (AA) at Bsl (A) and 8-Isoprostane Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) at 3-mo (B) With The Recovery (difference (Δ) between 3-mo and Bsl) of Peak Isometric Force (N/kg) of The Injured Limb. (A) Correlation between plasma AA (μM) data at Bsl and the recovery (i.e., 3-mo minus Bsl) in peak isometric force (N/kg) of the injured (INJ) limb for all subjects (PL and AO). Significant correlation between Bsl plasma AA and the recovery in peak isometric force of the INJ limb (r = 0.59, P = 0.006). (B) Pooled subject (PL, ●, and AO, ○, n = 20) data revealed an inverse correlation (r = -0.76; P = 0.007, solid regression line (—)) between plasma 8-iso-$PGF_{2\alpha}$ concentrations and the recovery of peak isometric force of the INJ limb. Additionally, the recovery of peak isometric force of the INJ limb was significantly correlated (r = -0.80, P = 0.005, dashed regression line (– – –)) with

plasma 8-iso-$PGF_{2\alpha}$ concentrations within the AO but not the PL group ($r = -0.14$, $P = 0.72$).

A.



B.



4.14. The Relationship Between The Interleukin (IL)-6:IL-10 Concentration Ratio With (A) The Peak Isometric Force (N/kg) of The Injured (INJ) ACL Limb and (B) Plasma 8-Isoprostane Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) at 3-mo Post-Surgery. (A) The IL-6:IL-10 ratio and peak isometric force of the INJ limb were significantly correlated ($r = 0.81$, $P = 0.005$) within the AO but not the PL group ($r = -0.10$, $P = 0.73$; data not shown). (B)

Plasma 8-iso-$PGF_{2\alpha}$ and the IL-6:IL-10 ratio were significantly correlated ($r = -0.77$, $P = 0.009$) within the AO but not the PL group ($r = -0.28$, $P = 0.43$; data not shown).

Tables

4.1. General Subject Characteristics.

	PL		AO	
	Bsl	3-mo	Bsl	3-mo
Body mass (kg)	90.0 ± 4.9	90.8 ± 4.9	89.8 ± 7.4	89.5 ± 7.3
Body mass index (BMI; kg/m^2)	29.7 ± 2.0	30.0 ± 2.0	28.9 ± 2.1	28.8 ± 2.1
Serum cholesterol (mmol/L)	4.6 ± 0.9	4.4 ± 0.2	4.8 ± 0.4	4.1 ± 0.3
Serum triglycerides (mmol/L)	1.8 ± 0.2	1.8 ± 0.4	2.0 ± 0.6	1.8 ± 0.3

Data presented as mean ± SEM (PL, *n* = 10; AO, *n* = 10). AO, antioxidant treated subjects. PL, placebo treated subjects. Bsl, baseline.

4.2. Plasma Malondialdehyde (MDA; μM) and Myeloperoxidase (MPO; ng/mL) Were Unaffected 3-mo Post-Surgery or By Antioxidant Supplementation.

	MDA		MPO	
	PL	AO	PL	AO
Bsl	0.94 ± 0.05	0.95 ± 0.05	50.9 ± 9.0	48.9 ± 14.1
Pre	1.04 ± 0.09	1.01 ± 0.03	58.3 ± 10.0	27.8 ± 7.1
90-min	1.17 ± 0.09	1.26 ± 0.06	50.0 ± 12.3	48.5 ± 12.2
72-h	1.00 ± 0.07	1.05 ± 0.08	45.9 ± 11.5	36.7 ± 11.6
7-d	0.94 ± 0.07	1.08 ± 0.07	36.5 ± 10.0	27.3 ± 5.1
3-mo	0.86 ± 0.06	0.97 ± 0.07	58.6 ± 9.9	64.2 ± 11.3

Data presented as mean ± SEM (PL, *n* = 10; AO, *n* = 10). AO, antioxidant treated subjects. PL, placebo treated subjects. Bsl, baseline. Pre, pre-surgery.

4.3. Plasma Cytokines (pg/mL) and Serum hsCRP (mg/L) Were Unaffected 3-mo Post-Surgery or By Antioxidant Supplementation.

	PL		AO	
	Bsl	3-mo	Bsl	3-mo
IL-10	5.4 (4.3, 7.2)	6.2 (5.7, 10.0)	8.7 (5.0, 21.6)	12.6 (5.3, 25.4)
IL-6	14.4 (6.4, 34.2)	14.5 (6.8, 30.6)	17.0 (8.8, 50.0)	36.2 (10.6, 64.2)
TNF-α	1.8 (1.0, 7.0)	2.3 (1.3, 10.3)	2.6 (1.0, 10.9)	3.2 (2.2, 10.7)
hsCRP	2.8 (1.8, 4.6)	2.6 (1.9, 3.6)	3.1 (2.0, 5.3)	2.9 (1.8, 3.6)

Data presented as median (inter-quartile range) (PL, n = 10; AO, n = 10).

CHAPTER 5. CONCLUSIONS

The purpose of this project was to test the hypothesis that vitamin E and C supplementation would ameliorate muscle dysfunction following ACL surgery by attenuating the increase in circulating and local mediators of muscle atrophy and weakness. To test this hypothesis we constructed four specific aims that were temporally designed around ischemia-reperfusion injury, limb disuse (or immobilization) and early (3-mo) post-surgery weakness following ACL surgery.

Specific Aim #1 was to identify circulating biomarkers of oxidative stress and inflammatory cytokines, and the influence of vitamin E and C supplementation on these mediators of muscle dysfunction following the ischemia-reperfusion conditions associated with ACL surgery. Vitamin E and C supplementation significantly increased plasma α-T and AA, and decreased γ-T concentrations. Following the localized arthroscopic repair of a ruptured ACL and tourniquet removal, we found a significant increase in circulating oxidative stress (lipid peroxidation), a marker of muscle damage and a pro-inflammatory cytokine several days after ACL reconstruction. Moreover, vitamin E and C supplementation ameliorated the increase of an anti-inflammatory cytokine and the depression of a pro-to-anti-inflammatory cytokine ratio immediately following ACL reconstruction. Based on these findings, we conclude that there is a systemic inflammatory cytokine and oxidative stress response immediately following ACL surgery, and that vitamin E and C supplementation provided protection against immuno-suppression but not oxidative stress.

Specific Aims #2 and #3 were to identify inflammatory-derived oxidative stress, inflammatory cytokine and inducible nitric oxide synthase expression in the musculature of the ACL injured limb prior to and following the limb disuse phase post-ACL surgery, and the influence of vitamin E and C supplementation on these mediators of muscle atrophy. There were no significant differences in muscle size (i.e., fiber cross-sectional areas) across time or between treatment groups. Compared to the pre-surgery, there was a significant increase in iNOS and MPO in the AO compared to that of the PL group. We conclude that short-term vitamin E and C supplementation does not appear to be beneficial on muscle size several days after surgery, and may exacerbate the infiltration of inflammatory cells.

Specific Aim #4 was to identify circulating mediators of muscle dysfunction, and the strength and strength recovery of the injured limb following an ACL injury and surgery; and the influence of vitamin E and C supplementation on these parameters. By 3-mo post-surgery, circulating mediators, strength and strength recovery of repaired limb were not significantly different between treatment groups. However, unlike the PL group, the injured limb in the AO group did not display a significant increase in peak isometric force from pre- to 3-mo post-surgery. We conclude that short-term vitamin E and C supplementation was not beneficial, but possibly, detrimental on the strength recovery following ACL surgery.

Overall Perspective and Significance

In summary, vitamin E and C supplementation provided protection against immuno-suppression, enhanced inflammatory cell infiltration into the musculature of the injured limb, was ineffective on circulating oxidative stress and muscle fiber size, and possibly

impaired strength recovery following ACL surgery. We interpret our data as suggesting that the short-term vitamin E and C supplementation may blunt the anti-inflammatory cytokine response and enhance the infiltration of inflammatory cells that impair the strength recovery of the injured ACL limb following surgery. We conclude that although antioxidant supplementation protected against immuno-suppression, a response that would be indicative of a better prognosis, it did not protect against muscle dysfunction and potentially may have contributed to dysfunction. Clearly, additional studies are warranted.

ABBREVIATIONS

AA, ascorbic acid; ACL, anterior cruciate ligament; α-T, α-tocopherol; AO, antioxidant; Bsl, baseline; Bwt, body weight; CK, creatine kinase; DMSO, dimethyl-sulfoxide; 8-iso-$PGF_{2\alpha}$, 8-isoprostane prostaglandin $F_{2\alpha}$; eNOS, endothelial nitric oxide synthase; γ-T, γ-tocopherol; GR, group (PL & AO data); Ht, height; HO-1, heme oxygenase-1; hsCRP, high-sensitivity C-reactive protein; HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; HNE, hydroxynonenal; iNOS, inducible nitric oxide synthase; INJ, injured limb; IFN-γ, interferon-γ; IL, interleukin; MDA, malondialdehyde; MFI, median fluorescence intensity; MPO, myeloperoxidase; NK, natural killer; nNOS, neuronal nitric oxide synthase; N, Newton; NO•, nitric oxide; NI, non-injured; NSAID, non-steroidal anti-inflammatory drugs; NWB, non-weight bearing; NF-kB, nuclear factor-kappa B; $ONOO^-$, peroxynitrite; PL, placebo; Pre, pre-surgery; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RNS, reactive nitrogen species; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide; SL, single leg; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-α; Th1, type 1 helper T cells; Th2, type 2 helper T cells; UA, uric acid; WB, weight-bearing.

BIBLIOGRAPHY

[1] Adams V., Nehrhoff B., Spate U., Linke A., Schulze P.C., Baur A. et al. Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: an in vitro and in vivo study. *Cardiovasc Res* ;**54**:95-104;2002

[2] Adolfsson O., Huber B.T., Meydani S.N. Vitamin E-enhanced IL-2 production in old mice: naive but not memory T cells show increased cell division cycling and IL-2-producing capacity. *J Immunol* ;**167**:3809-17;2001

[3] Akira S., Taga T., Kishimoto T. Interleukin-6 in biology and medicine. *Adv Immunol* ;**54**:1-78;1993

[4] Aldemir O., Celebi H., Cevik C., Duzgun E. The effects of propofol or halothane on free radical production after tourniquet induced ischemia-reperfusion injury during knee arthroplasty. *Acta Anaesthesiol Scand* ;**45**:1221-5;2001

[5] Allaouchiche B., Debon R., Goudable J., Chassard D., Duflo F. Oxidative stress status during exposure to propofol, sevoflurane and desflurane. *Anesth Analg* ;**93**:981-5;2001

[6] Alloatti G., Penna C., Mariano F., Camussi G. Role of NO and PAF in the impairment of skeletal muscle contractility induced by TNF-α. *Am J Physiol Regul Integr Comp Physiol* ;**279**:R2156-R2163;2000

[7] Anderson J.L., Lamb S.E., Barker K.L., Davies S., Dodd C.A., Beard D.J. Changes in muscle torque following anterior cruciate ligament reconstruction. *Acta Orthop Scand* ;**73**:546-52;2002

[8] Andrade F.H., Reid M.B., Allen D.G., Westerblad H. Effect of nitric oxide on single skeletal muscle fibres from the mouse. *J Physiol (Lond)* ;**509**:577-86;1998

[9] Andrade F.H., Reid M.B., Westerblad H. Contractile response to low peroxide concentrations: myofibrillar calcium sensitivity as a likely target for redox-modulation of skeletal muscle function. *FASEB J* ;**15**:309-11;2001

[10] Andrade F.H., Reid M.R., Allen D.G., Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol (Lond)* ;**509**:565-75;1998

[11] Appell H.J. Morphology of immobilized skeletal muscle and the effects of a pre- and postimmobilization training program. *Int J Sports Med* ;**7**:6-12;1986

[12] Appell H.J., Ascensao A., Natsis K., Michael J., Duarte J.A.R. Signs of necrosis and inflammation do not support the concept of apoptosis as the

predominant mechanism during early atrophy in immobilized muscle. *Basic Appl Myol* ;**14**:191-6;2004

[13] Appell H.J., Duarte J.A.R., Gloser S., Remiao F., Carvalho F., Bastos M.L. et al. Administration of tourniquet. II. Prevention of postischemic oxidative stress can reduce muscle edema. *Arch Orthop Trauma Surg* ;**116**:101-6;1997

[14] Appell H.J., Duarte J.A.R., Soares J.M.C. Supplementation of vitamin E may attenuate skeletal muscle immobilization atrophy. *Int J Sports Med* ;**18**:157-60;1997

[15] Appell H.J., Gloser S., Duarte J.A.R., Zellner A., Soares J.M.C. Skeletal muscle damage during tourniquet-induced ischaemia. *Eur J Appl Physiol* ;**67**:342-7;1993

[16] Appell H.J., Gloser S., Soares J.M.C., Duarte J.A.R. Structural alterations of skeletal muscle induced by ischemia and reperfusion. *Basic Appl Myol* ;**9**:262-8;1999

[17] Arangio G.A., Chen C., Kalady M., Reed J.F. Thigh muscle size and strength after anterior cruciate ligament reconstruction and rehabilitation. *JOSPT* ;**26**:238-43;1997

[18] Arbogast S., Smith J., Matuszczak Y., Hardin B., Moylan J., Smith J.D. et al. Bowman-Birk Inhibitor Concentrate Prevents Atrophy, Weakness, and Oxidative Stress in Soleus Muscle of Hindlimb-Unloaded Mice. *J Appl Physiol* ;**102**:956-64;2006

[19] Arciero R.A., Scoville C.R., Hayda R.A., Snyder R.J. The effect of tourniquet use in anterior cruciate ligament reconstruction. A prospective, randomized study. *Am J Sports Med* ;**24**:758-64;1996

[20] Artacho-Perula E., Roldan-Villalobos R., Vaamonde-Lemos R. Capillary and fiber size interrelationships in regenerating rat soleus muscle after ischemia: a quantitative study. *Acta Ant* ;**142**:70-6;1991

[21] Arvidsson I., Eriksson E., Haggmark T., Johnson R.J. Isokinetic thigh muscle strength after ligament reconstruction in the knee joint: results from a 5-10 year follow-up after reconstructions of the anterior cruciate ligament in the knee joint. *Int J Sports Med* ;**2**:7-11;1981

[22] Arvidsson I., Arvidsson H., Eriksson E., Jansson E. Prevention of quadriceps wasting after immobilization: An evaluation of the effect of electrical stimulation. *Orthopedics* ;**9**:1519-28;1986

[23] Balon T.W., Nadler J.L. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol* ;**77**:2519-21;1994

[24] Barclay J.K., Hansel M. Free radicals may contribute to oxidative skeletal muscle fatigue. *Can J Physiol Pharmacol* ;**69**:279-84;1991

[25] Barker J.E., Knight K.R., Romeo R., Hurley J.V., Morrison W.A., Stewart A.G. Targeted disruption of the nitric oxide synthase 2 gene protects against ischaemia/reperfusion injury to skeletal muscle. *J Pathol* ;**194**:109-15;2001

[26] Barker T., Leonard S.W., Trawick R.H., Martins T.B., Kjeldsberg C.R., Hill H.R. et al. Modulation of inflammation by vitamin E and C supplementation prior to anterior cruciate ligament surgery. *Free Radic Biol Med* ;**46**:599-606;2009

[27] Barker T., Traber M.G. From animals to humans: evidence linking oxidative stress as a causative factor in muscle atrophy. *J Physiol (Lond)* ;**583**:421-2;2007

[28] Barker T., Traber M.G. Response to the Letter to the Editor by Rennie et al. *J Physiol (Lond)* ;**586**:309-10;2008

[29] Barreiro E., Coronell C., Lavina B., Ramirez-Sarmiento A., Orozco-Levi M., Gea J. Aging, sex differences, and oxidative stress in human respiratory and limb muscles. *Free Radic Biol Med* ;**41**:797-809;2006

[30] Bartali B., Frongillo E.A., Guralnik J.M., Stipanuk M.H., Allore H.G., Cherubini A. et al. Serum micronutrient concentrations and decline in physical function among older persons. *JAMA* ;**299**:308-15;2008

[31] Bartoccioni E., Michaelis D., Hohlfeld R. Constitutive and cytokine-induced production of interleukin-6 by human myoblasts. *Immunol Lett* ;**42**:135-8;1994

[32] Basu S. Metabolism of 8-iso-prostaglandin F2alpha. *FEBS Lett* ;**428**:32-6;1998

[33] Beckman J.S., Beckman T.W., Chen J., Marshall P.A., Freeman B.A. Apparent hydroxyl radical production by peroxynitrite; implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* ;**87**:1620-4;1990

[34] Bergman M., Salman H., Djaldetti M., Fish L., Punsky I., Bessler H. In vitro immune response of human peripheral blood cells to vitamins C and E. *J Nutr Biochem* ;**15**:45-50;2004

[35] Betters J.L., Criswell D.S., Shanely R.A., Gammeren D.V., Falk D., DeRuisseau K.C. et al. Trolox attenuates mechanically ventilation-induced diaphragmatic dysfunction aand proteolysis. *American Journal of Respiratory and Critical Care Medicine* ;**170**:1179-84;2004

[36] Ble A., Cherubini A., Volpato S., Bartali B., Walston J.D., Windham B.G. et al. Lower plasma vitamin E levels are associated with the frailty syndrome: the InCHIANTI study. *J Gerontol A Biol Sci Med Sci* ;**61**:278-83;2006

[37] Boczkowski J., Lanone S., Ungureanu-Longrois D., Danialou G., Fournier T., Mechighel M.A.w.t.t.a.o.M.P. Induction of Diaphragmatic Nitric Oxide Synthase after Endotoxin Administration in Rats . Role on Diaphragmatic Contractile Dysfunction. *J Clin Invest* ;**98**:1550-9;1996

[38] Bodine S.C., Latres E., Baumhueter S., Lai V.K.M., Nunez L., Clarke B.A. et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* ;**294**:1704-8;2001

[39] Bogdan C., Paik J., Vodovotz Y., Nathan C. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *J Biol Chem* ;**267**:23301-8;1992

[40] Boom W.H., Liano D., Abbas A.K. Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin 4- and interleukin 2-producing T cell clones on resting B lymphocytes. *J Exp Med* ;**167**:1350-63;1988

[41] Booth F.W. Time course of muscular atrophy during immobilization of hindlimbs in rats. *J Appl Physiol* ;**43**:656-61;1977

[42] Booth F.W., Kelso J.R. Production of rat muscle atrophy by cast fixation. *J Appl Physiol* ;**34**:404-6;1973

[43] Booth F.W., Seider M.J. Early changes in skeletal muscle protein synthesis after limb immobilization of rats. *J Appl Physiol* ;**47**:974-7;1979

[44] Booth F.W., Seider M.J. Recovery of skeletal muscle after 3 mo of hindlimb immobilization in rats. *J Appl Physiol* ;**47**:435-9;1979

[45] Bozkurt A.K. Alpha-tocopherol (vitamin E) and iloprost attenuate reperfusion injury in skeletal muscle ischemia/reperfusion injury. *J Cardiovasc Surg (Torino)* ;**43**:693-6;2002

[46] Broussard S.R., McCusker R.H., Novakofski J.E., Strle K., Shen W.H., Johnson R.W. et al. IL-1beta impairs insulin-like growth factor i-induced differentiation and downstream activation signals of the insulin-like growth factor i receptor in myoblasts. *J Immunol* ;**172**:7713-20;2004

[47] Bruno R.S., Leonard S.W., Atkinson J., Montine T.J., Ramakrishnan R., Bray T.M. et al. Faster plasma vitamin E disappearance in smokers is normalized by vitamin C supplementation. *Free Radic Biol Med* ;**40**:689-97;2006

[48] Bruton J.D., Place N., Yamada T., Silva J.P., Andrade F.H., Dahlstedt A.J. et al. Reactive oxygen species and fatigue-induced prolonged low-frequency

force depression in skeletal muscle fibres of rats, mice and SOD2 overexpressing mice. *J Physiol (Lond)* ;**586**:175-84;2008

[49] Bruunsgaard H., Bjerregaard E., Schroll M., Pedersen B.K. Muscle strength after resistance training is inversely correlated with baseline levels of soluble tumor necrosis factor receptors in the oldest old. *J Am Geriatr Soc* ;**52**:237-41;2004

[50] Buck M., Chojkier M. Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. *The EMBO Journal* ;**15**:1753-65;2005

[51] Burton G.W., Ingold K.U. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc Chem Res* ;**19**:194-201;1986

[52] Bushell A., Klenerman L., Davies H., Grierson I., Jackson M.J. Ischemia-reperfusion-induced muscle damage. *Acta Orthop Scand* ;**67**:393-8;1996

[53] Bussolati B., Rollino C., Mariano F., Quarello F., Camussi G. IL-10 stimulates production of platelet-activating factor by monocytes of patients with active systemic lupus erythematosus (SLE). *Clin Exp Immunol* ;**122**:471-6;2000

[54] Buttemeyer R., Phillipp A.W., Mall J.W., Freider B.G., Scheller F.W., Lisdat F. In vivo measurement of oxygen-derived free radicals during reperfusion injury. *Microsurgery* ;**22**:108-13;2002

[55] Callahan L.A., She Z.W., Nosek T.M. Superoxide, hydroxyl radical, and hydrogen peroxide effects on single-diaphragm fiber contractile apparatus. *J Appl Physiol* ;**90**:45-54;2001

[56] Cameron M., Buchgraber A., Passler H., Vogt M., Thonar E., Fu F. et al. The natural history of the anterior cruciate ligament-deficient knee. Changes in synovial fluid cytokine and keratan sulfate concentrations. *Am J Sports Med* ;**25**:751-4;1997

[57] Carden D.L., Korthuis R.J. Protesae inhibition attenuates microvascular dysfunction in postischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**271**:H1947-H1952;1996

[58] Carr A., Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J* ;**13**:1007-24;1999

[59] Carvalho A.J., Hollett P., McKee N.H. Recovery of synergistic skeletal muscle function following ischemia. *J Surg Res* ;**59**:527-33;1995

[60] Castell J.V., Gomez-Lechon M.J., David M., Hirano T., Kishimoto T., Heinrich P.C. Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the

synthesis of acute phase proteins in human hepatocytes. *FEBS Lett* ;**232**:347-50;1988

[61] Cesari M., Pahor M., Bartali B., Cherubini A., Penninx B.W.J.H., Williams G.R. et al. Antioxidants and physical performance in elderly persons: the Invecchiare in Chianti (InCHIANTI) study. *Am J Clin Nutr* ;**79**:289-94;2004

[62] Cesari M., Penninx B.W., Pahor M., Lauretani F., Corsi A.M., Rhys W.G. et al. Inflammatory markers and physical performance in older persons: the InCHIANTI study. *J Gerontol A Biol Sci Med Sci* ;**59**:242-8;2004

[63] Chen C.H., Chuang J.H., Liu K., Chan J.Y. Nitric oxide triggers delayed anesthetic preconditioning-induced cardiac protection via activation of nuclear factor-kappaB and upregulation of inducible nitric oxide synthase. *Shock* ;**30**:241-9;2008

[64] Chen L.-E., Seaber A.V., Nasser R.M., Stamler J.S., Urbaniak J.R. Effect of *S*-nitro-*N*-acetylcysteine on contractile function of reperfused skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* ;**274**:R822-R829;1998

[65] Chen L.-E., Silver W.P., Seaber A.V., Korompilias A.V., Urbaniak J.R. Effects of dexamethasone on the contractile function of reperfused skeletal muscle. *Microsurgery* ;**17**:313-20;1996

[66] Cher D.J., Mosmann T.R. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. *J Immunol* ;**138**:3688-94;1987

[67] Close G.L., Ashton T., Cable T., Doran D., MacLaren D.P. Eccentric exercise, isokinetic muscle torque and delayed onset muscle soreness: the role of reactive oxygen species. *Eur J Appl Physiol* ;**91**:615-21;2004

[68] Coffman R.L., Seymour B.W., Lebman D.A., Hiraki D.D., Christiansen J.A., Shrader B. et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol Rev* ;**102**:5-28;1988

[69] Daftarian P.M., Kumar A., Kryworuchko M., Diaz-Mitoma F. IL-10 production is enhanced in human T cells by IL-12 and IL-6 and in monocytes by tumor necrosis factor-alpha. *J Immunol* ;**157**:12-20;1996

[70] Daniel D.M., Lumkong G., Stone M.L., Pedowitz R.A. Effects of tourniquet use in anterior cruciate ligament reconstruction. *Arthroscopy* ;**11**:307-11;1995

[71] Darnley G.M., Duke A.M., Steele D.S., MacFarlane N.G. Effects of reactive oxygen species on aspects of excitation-contraction coupling in chemically skinned rabbit diaphragm muscle fibres. *Exp Physiol* ;**86**:-161;2001

[72] Davies K.J.A. Protein modification by oxidants and the role of proteolytic enzymes. *Biochem Soc Trans* ;**21**:346-53;1993

[73] Davies K.J.A. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* ;**83**:301-10;2001

[74] Daynes R.A., Araneo B.A., Ershler W.B., Maloney C., Li G.Z., Ryu S.Y. Altered regulation of IL-6 production with normal aging. Possible linkage to the age-associated decline in dehydroepiandrosterone and its sulfated derivative. *J Immunol* ;**150**:5219-30;1993

[75] Deblier I., Sadowska A.M., Janssens A., Rodrigus I., DeBacker W.A. Markers of inflammation and oxidative stress in patients undergoing CABG with CPB with and without ventilation of the lungs: a pilot study. *Interact Cardiovasc Thorac Surg* ;**5**:387-91;2006

[76] DeForge L.E., Fantone J.C., Kenney J.S., Remick D.G. Oxygen radical scavengers selectively inhibit interleukin 8 production in human whole blood. *J Clin Invest* ;**90**:2123-9;1992

[77] DeForge L.E., Preston A.M., Takeuchi E., Kenney J., Boxer L.A., Remick D.G. Regulation of interleukin 8 gene expression by oxidant stress. *J Biol Chem* ;**268**:25568-76;1993

[78] Dehoux M., Gobier C., Lause P., Bertrand L., Ketelslegers J.M., Thissen J.P. IGF-I does not prevent myotube atrophy caused by proinflammatory cytokines despite activation of Akt/Foxo and GSK-3beta pathways and inhibition of atrogin-1 mRNA. *Am J Physiol Endocrinol Metab* ;**292**:E145-E150;2007

[79] Del Prete G., De Carli M., Almerigogna F., Giudizi M.G., Biagiotti R., Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* ;**150**:353-60;1993

[80] Devaraj S., Jialal I. Alpha tocopherol supplementation decreases serum C-reactive protein and monocyte interleukin-6 levels in normal volunteers and type 2 diabetic patients. *Free Radic Biol Med* ;**29**:790-2;2000

[81] Devaraj S., Leonard S., Traber M.G., Jialal I. Gamma-tocopherol supplementation alone and in combination with alpha-tocopherol alters biomarkers of oxidative stress and inflammation in subjects with metabolic syndrome. *Free Radic Biol Med* ;**44**:1203-8;2008

[82] Devaraj S., Tang R., Adams-Huet B., Harris A., Seenivasan T., de Lemos J.A. et al. Effect of high-dose alpha-tocopherol supplementation on biomarkers of oxidative stress and inflammation and carotid atherosclerosis in patients with coronary artery disease. *Am J Clin Nutr* ;**86**:1392-8;2007

[83] Diaz P.T., Brownstein E., Clanton T.L. Effects of N-acetylcysteine on in vitro diaphragam function are temperature dependent. *J Appl Physiol* ;**77**:2434-9;1994

[84] Diaz P.T., Julian M.W., Wewers M.D., Clanton T.L. Tumor nectrosis factor and endotoxin do not directly affect in vitro diaphragm function. *Am Rev Respir Dis* ;**148**:281-7;1993

[85] Diaz P.T., She Z.W., Davis W.B., Clanton T.L. Hydroxylation of salicylate by the in vitro diaphragm: evidence for hydroxyl radical protuction during fatigue. *J Appl Physiol* ;**75**:540-5;1993

[86] Dietrich M., Block G., Benowitz N.L., Morrow J.D., Hudes M., Jacob P., III et al. Vitamin C supplementation decreases oxidative stress biomarker f2-isoprostanes in plasma of nonsmokers exposed to environmental tobacco smoke. *Nutr Cancer* ;**45**:176-84;2003

[87] Dietrich M., Block G., Hudes M., Morrow J.D., Norkus E.P., Traber M.G. et al. Antioxidant supplementation decreases lipid peroxidation biomarker F(2)-isoprostanes in plasma of smokers. *Cancer Epidemiol Biomarkers Prev* ;**11**:7-13;2002

[88] Dinarello C.A. Proinflammatory cytokines. *Chest* ;**118**:503-8;2000

[89] Divangahi M., Demoule A., Danialou G., Yahiaoui L., Bao W., Xing Z. et al. Impact of IL-10 on diaphragmatic cytokine expression and contractility during Pseudomonas Infection. *Am J Respir Cell Mol Biol* ;**36**:504-12;2007

[90] Dokka S., Shi X., Leonard S., Wang L., Castranova V., Rojanasakul Y. Interleukin-10-mediated inhibition of free radical generation in macrophages. *Am J Physiol Lung Cell Mol Physiol* ;**280**:L1196-L1202;2001

[91] Dominguez-Rodriguez A., Abreu-Gonzalez P., de la R.A., Vargas M., Ferrer J., Garcia M. Role of endogenous interleukin-10 production and lipid peroxidation in patients with acute myocardial infarction treated with primary percutaneous transluminal coronary angioplasty, interleukin-10 and primary angioplasty. *Int J Cardiol* ;**99**:77-81;2005

[92] Domzig W., Stadler B.M., Herberman R.B. Interleukin 2 dependence of human natural killer (NK) cell activity. *J Immunol* ;**130**:1970-3;1983

[93] Dorion D., Zhong A., Chiu C., Forrest C.R., Boyd B., Pang C.Y. Role of xanthine oxidase in reperfusion injury of ischemic skeletal muscles in the pig and human. *J Appl Physiol* ;**75**:246-55;1993

[94] Duarte J.A.R., Gloser S., Remiao F., Carvalho F., Bastos M.L., Soares J.M.C. et al. Administration of tourniquet. I. are edema and oxidative stress related to

each other and to the duration of ischemia in reperfused skeletal muscle? *Arch Orthop Trauma Surg* ;**116**:97-100;1997

[95] Dupouy V.M., Ferre P.J., Uro-Coste E., Lefebvre H.P. Time course of COX-1 and COX-2 expression during ischemia-reperfusion in rat skeletal muscle. *J Appl Physiol* ;**100**:233-9;2006

[96] Edstrom L. Selective atrophy of red muscle fibres in the quadriceps in long-standing knee-joint dysfunction. Injuries to the anterior cruciate ligament. *J Neurol Sci* ;**11**:551-8;1970

[97] Elmqvist L.-G., Lorentzon R., Johansson C., Langstrom M., Fagerlund M., Fugl-Meyer A.R. Knee extensor muscle function before and after reconstruction of anterior cruciate ligament tear. *Scand J Rehab Med Suppl* ;**21**:131-9;1989

[98] Eriksson K., Hamberg P., Jansson E., Larsson H., Shalabi A., Wredmark T. Semitendinosus muscle in anterior cruciate ligament surgery: morphology and function. *Arthroscopy* ;**17**:808-17;2001

[99] Eskdale J., Kube D., Gallagher G. A second polymorphic dinucleotide repeat in the 5' flanking region of the human IL10 gene. *Immunogenetics* ;**45**:82-3;1996

[100] Evans T.J., Buttery L.D., Carpenter A., Springall D.R., Polak J.M., Cohen J. Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. *Proc Natl Acad Sci USA* ;**93**:9553-8;1996

[101] Fano G., Mecocci P., Vecchiet J., Belia S., Fulle S., Polidori M.C. et al. Age and sex influence on oxidative damage and functional status in human skeletal muscle. *J Muscle Res Cell Motil* ;**22**:345-51;2001

[102] Fantini G.A., Yoshioka T. Deferoxamine prevents lipid peroxidation aand attenuates reoxygenation injury in postischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**264**:H1953-H1959;1993

[103] Fermor B., Urban J., Murray D., Pocock A., Lim E., Francis M. et al. Proliferation and collagen synthesis of human anterior cruciate ligament cells *in vitro*: effects of ascorbate-2-phosphate, dexamethasone and oxygen tension. *Cell Biol Int* ;**22**:635-40;1998

[104] Ferrari R.P., Battiston B., Brunelli G., Casella A., Calmi L. The role of allopurinol in preventing oxygen free radical injury to skeletal muscle and endothelial cells after ischemia-reperfusion. *J Recon Microsurg* ;**12**:447-50;1996

[105] Fiorentino D.F., Bond M.W., Mosmann T.R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* ;**170**:2081-95;1989

[106] Fiorentino D.F., Zlotnik A., Mosmann T.R., Howard M., O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* ;**147**:3815-22;1991

[107] Fiorentino D.F., Zlotnik A., Vieira P., Mosmann T.R., Howard M., Moore K.W. et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* ;**146**:3444-51;1991

[108] Fish J.S., McKee N.H., Pynn B.R., Kuzon W.M., Plyley M.J. Isometric contractile function recovery following tourniquet ischemia. *J Surg Res* ;**47**:365-70;1989

[109] Fitts R.H., Metzger J.M., Riley D.A., Unsworth B.R. Models of disuse: a comparison of hindlimb suspension and immobilization. *J Appl Physiol* ;**60**:1946-53;1986

[110] Flores E.A., Bistrian B.R., Pomposelli J.J., Dinarello C.A., Blackburn G.L., Istfan N.W. Infusion of tumor necrosis factor/cachectin promotes muscle catabolism in the rat: A synergistic effect with interleukin 1. *J Clin Invest* ;**83**:1614-22;1989

[111] Forbes T.L., Carson M., Harris K.A., DeRose G., Jamieson W.G., Potter R.F. Skeletal muscle injury induced by ischemia-reperfusion. *JCC* ;**38**:56-63;1995

[112] Formigli L., Lombardo L.D., Adembri C., Brunelleschi S., Ferrari E., Novelli G.P. Neutrophils as mediators of human skeletal muscle ischemia-reperfusion syndrome. *Hum Path* ;**23**:627-34;1992

[113] Formigli L., Manneschi L.I., Tani A., Gandini E., Adembri C., Pratesi C. et al. Vitamin E prevents neutrophil accumulation and attenuates tissue damage in ischemic-reperfused human skeletal muscle. *Histol Histopathol* ;**12**:663-9;1997

[114] Frandsen U., Lopez-Figueroa M., Hellsten Y. Localization of nitric oxide synthase in human skeletal muscle. *Biochem Biophys Res Commun* ;**227**:888-93;1996

[115] Frei B., England L., Ames B.N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci USA* ;**86**:6377-81;1989

[116] Frost R.A., Nystrom G.J., Lang C.H. Tumor necrosis factor-alpha decreases insulin-like growth factor-I messenger ribonucleic acid expression in C2C12 myoblasts via a Jun N-terminal kinase pathway. *Endocrinology* ;**144**:1770-9;2003

[117] Gaines G.C., Welborn M.B., Moldawer L.L., Huber T.S., Harward T.R.S., Seeger J.M. Attenuation of skeletal muscle ischemia/reperfusion injury by inhibition of tumor necrosis. *J Vasc Surg* ;**29**:370-6;1999

[118] Galler S., Hilber K., Gosbesberger A. Effects of nitric oxide on force-generating proteins of skeletal muscle. *Pflugers Arch-Eur J Physiol* ;**434**:242-5;1997

[119] Gan W.Q., Man S.F., Senthilselvan A., Sin D.D. Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax* ;**59**:574-80;2004

[120] Geiger T., Andus T., Klapproth J., Hirano T., Kishimoto T., Heinrich P.C. Induction of rat acute-phase proteins by interleukin 6 in vivo. *Eur J Immunol* ;**18**:717-21;1988

[121] Georganas C., Liu H., Perlman H., Hoffmann A., Thimmapaya B., Pope R.M. Regulation of IL-6 and IL-8 expression in rheumatoid arthritis synovial fibroblasts: the dominant role for NF-kappa B but not C/EBP beta or c-Jun. *J Immunol* ;**165**:7199-206;2000

[122] Gerber C., Hoppeler H., Claassen H., Robotti G., Zehnder R., Jakob R.P. et al. The lower-extremity musculature in chronic symptomatic instability of the anterior cruciate ligament. *J Bone Joint Surg (Am)* ;**67-A**:1034-43;1985

[123] Gerber J.P., Marcus R.L., Dibble L.E., Greis P.E., Burks R.T., LaStayo P.C. Effects of Early Progressive Eccentric Exercise on Muscle Structure After Anterior Cruciate Ligament Reconstruction. *J Bone Joint Surg (Am)* ;**89**:559-70;2007

[124] Gianni P., Jan K.J., Douglas M.J., Stuart P.M., Tarnopolsky M.A. Oxidative stress and the mitochondrial theory of aging in human skeletal muscle. *Exp Gerontol* ;**39**:1391-400;2004

[125] Glynn P., Coakley R., Kilgallen I., Murphy N., O'Neill S. Circulating interleukin 6 and interleukin 10 in community acquired pneumonia. *Thorax* ;**54**:51-5;1999

[126] Gogos C.A., Drosou E., Bassaris H.P., Skoutelis A. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* ;**181**:176-80;2000

[127] Goodman M.N. Tumor necrosis factor induces skeletal muscle protein breakdown in rats. *Am J Physiol Endocrinol Metab* ;**260**:E727-E730;1991

[128] Goodman M.N. Interleukin-6 induces skeletal muscle protein breakdown in rats. *Proc Soc Exp Biol Med* ;**205**:182-5;1994

[129] Grant J.A., Mohtadi N.G.H., Maitland M.E., Zernicke R.F. Comparison of Home Versus Physical Therapy-Supervised Rehabilitation Programs After Anterior Cruciate Ligament Reconstruction: A Randomized Clinical Trial. *Am J Sports Med* ;**33**:1288-97;2005

[130] Greiwe J.S., Cheng B.O., Ubin D.C., arasheski K.E., emenkovich C.F. Resistance exercise decreases skeletal muscle tumor necrosis factor {alpha} in frail elderly humans. *FASEB J* ;**15**:475-82;2001

[131] Grisham M.B., Hernandez L.A., Granger D.N. Xanthine oxidasae and neutrophil infiltration in intestinaal ischemia. *AJP - Gastrointestinal and Liver Physiology* ;**251**:G567-G574;1986

[132] Grisotto P.C., do Santos A.C., Coutinho-Netto J., Cherri J., Piccinato C.E. Indicators of oxidative injury and alterations of the cell membrane in the skeletal muscle of rats submitted to ischemia and reperfusion. *J Surg Res* ;**92**:1-6;2000

[133] Grosser N., Abate A., Oberle S., Vreman H.J., Dennery P.A., Becker J.C. et al. Heme oxygenase-1 induction may explain the antioxidant profile of aspirin. *Biochem Biophys Res Commun* ;**308**:956-60;2003

[134] Guo Y., Jones W.K., Xuan Y.T., Tang X.L., Bao W., Wu W.J. et al. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. *Proc Natl Acad Sci USA* ;**96**:11507-12;1999

[135] Haddad F., Zaldivar F., Cooper D.M., Adams G.R. IL-6-induced muscle atrophy. *J Appl Physiol* ;**98**:911-7;2005

[136] Haddad J.J., Fahlman C.S. Redox- and oxidant-mediated regulation of interleukin-10: an anti-inflammatory, antioxidant cytokine? *Biochem Biophys Res Commun* ;**297**:163-76;2002

[137] Haggmark T., Jansson E., Eriksson E. Fiber type area and metabolic potential of the thigh muscle in man after knee surgery and immobilization. *Int J Sports Med* ;**2**:12-7;1981

[138] Halliwell B., Gutteridge J.M.C. Free Radicals in Biology and Medicine. New York: Oxford University Press; 1999.

[139] Halpner A.D., Handelman G.J., Harris J.M., Belmont C.A., Blumberg J.B. Protection of vitamin C of loss of vitamin E in cultured rat hepatocytes. *Arch Biochem Biophys* ;**359**:305-9;1998

[140] Hambrecht R., Adams V., Gielen S., Linke A., Mobius-Winkler S., Yu J. et al. Exercise intolerance in patients with chronic heart failure and increased expression of inducible nitric oxide synthase in the skeletal muscle. *J Am Coll Cardiol* ;**33**:174-9;1999

[141] Han S.N., Adolfsson O., Lee C.K., Prolla T.A., Ordovas J., Meydani S.N. Age and vitamin E-induced changes in gene expression profiles of T cells. *J Immunol* ;**177**:6052-61;2006

[142] Han S.N., Wu D., Ha W.K., Beharka A., Smith D.E., Bender B.S. et al. Vitamin E supplementation increases T helper 1 cytokine production in old mice infected with influenza virus. *Immunology* ;**100**:487-93;2000

[143] Hardin B.J., Campbell K.S., Smith J.D., Arbogast S., Smith J., Moylan J.S. et al. TNF-{alpha} acts via TNFR1 and muscle-derived oxidants to depress myofibrillar force in murine skeletal muscle. *J Appl Physiol* ;**104**:694-9;2008

[144] Harris K., Walker P.M., Mickle D.A.G., Harding R., Gatley R., Wilson G.J. et al. Metabolic response of skeletal muscle to ischemia. *Am J Physiol Heart Circ Physiol* ;**250**:H213-H220;1986

[145] Harrison J.E., Schultz J. Studies on the chlorinating activity of myeloperoxidase. *J Biol Chem* ;**251**:1371-4;1976

[146] Heunks L.M.A., Cody M.J., Geiger P.C., Dekhuijzen P.N.R., Sieck G.C. Nitric oxide impairs ca2+ activation and slows cross-bridge cycling kinetics in skeletal muscle. *J Appl Physiol* ;**91**:2233-9;2001

[147] Higuchi H., Shirakura K., Kimura M., Terauchi M., Shinozaki T., Watanabe H. et al. Changes in biochemical parameters after anterior cruciate ligament injury. *Int Orthop* ;**30**:43-7;2006

[148] Holm L., Esmarck B., Mizuno M., Hansen H., Suetta C., Holmich P. et al. The effect of protein and carbohydrate supplementation on strength training outcome of rehabilitation in ACL patients. *J Orthop Res* ;**24**:2114-23;2006

[149] Hong Y.L., Yeh S.L., Chang C.Y., Hu M.L. Total plasma malondialdehyde levels in 16 Taiwanese college students determined by various thiobarbituric acid tests and an improved high-performance liquid chromatography-based method. *Clin Biochem* ;**33**:619-25;2000

[150] Howard C., Ferrucci L., Sun K., Fried L.P., Walston J., Varadhan R. et al. Oxidative protein damage is associated with poor grip strength among older women living in the community. *J Appl Physiol* ;**103**:17-20;2007

[151] Huang H.Y., Appel L.J., Croft K.D., Miller E.R., III, Mori T.A., Puddey I.B. Effects of vitamin C and vitamin E on in vivo lipid peroxidation: results of a randomized controlled trial. *Am J Clin Nutr* ;**76**:549-55;2002

[152] Huda R., Solanki D.R., Mathru M. Inflammatory and redox responses to ischaemia/reperfusion in human skeletal muscle. *Clin Sci (Lond)* ;**107**:497-503;2004

[153] Huey K.A., Fiscus G., Richwine A.F., Johnson R.W., Meador B.M. In vivo vitamin E administration attenuates interleukin-6 and interleukin-1{beta} responses to an acute inflammatory insult in mouse skeletal and cardiac muscle. *Exp Physiol* ;**93**:1263-72;2008

[154] Idstrom J.-P., Soussi B., Elander A., Bylund-Fellenius A.-C. Purine metabolism after *in vivo* ischemia and reperfusion in rat skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**258**:H1668-H1673;1990

[155] Idstrom J.-P., Soussi B., Wanag E., Bylund-Fellenius A.-C. Analysis of purine nucleotides in muscle tissue by HPLC. *Scand J Clin Lab Invest* ;**50**:541-9;1990

[156] Ikemoto M., Nikawa T., Kano M., Kitano T., Watanabe C., Tanaka H. et al. Cysteine supplementation prevents unweighting-induced ubiquintination in association with redox regulation in rat skeletal muscle. *Biol Chem* ;**383**:715-21;2002

[157] Ikemoto M., Okamura Y., Kano M., Hirasak K., Tanaka R., Yamamoto T. et al. A relative high dose of vitamin E does not attenuate unweighting-induced oxidative stress and ubiquitination in rat skeletal muscle. *J Physiol Anthropol Appl Human Sci* ;**21**:257-63;2002

[158] Irie K., Uchiyama E., Iwaso H. Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. *The Knee* ;**10**:93-6;2003

[159] Jacobsen M.D., Pedowitz R.A., Oyama B.K., Tryon B., Gershuni D.H. Muscle functional deficits after tourniquet ischemia. *Am J Sports Med* ;**22**:372-7;1994

[160] Jagoe R.T., Goldberg A.F. What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? *Curr Opin Clin Nutr Metab Care* ;**4**:183-90;2001

[161] Jones S.W., Hill R.J., Krasney P.A., O'Conner B., Peirce N., Greenhaff P.L. Disuse atrophy and exercise rehabilitation in humans profoundly affects the expression of genes associated with the regulation of skeletal muscle mass. *FASEB J* ;**18**:1025-7;2004

[162] Jorgensen H.R.I. Myoglobin releasae after tourniquet ischemia. *Acta Orthop Scand* ;**58**:554-7;1987

[163] Judge A.R., Selsby J.T., Dodd S.L. Antioxidants attenuate oxidative damage in rat skeletal muscle during mild ischaemia. *Exp Physiol* ;**93**:479-85;2008

[164] Kahraman S., Kilinc K., Dal D., Erdem K. Propofol attenuates formation of lipid peroxides in tourniquet-induced ischemia-reperfusion. *Br J Anaesth* ;**78**:279-81;1997

[165] Kannus P., Jozsa L., Jarvinen T.L.N., Kvist M., Vieno T., Jarvinen T.A.H. et al. Free mobilization and low- to high-intensity exercise in immobilization-induced muscle atrophy. *J Appl Physiol* ;**84**:1418-24;1998

[166] Kasid A., Director E.P., Rosenberg S.A. Induction of endogenous cytokine-mRNA in circulating peripheral blood mononuclear cells by IL-2 administration to cancer patients. *J Immunol* ;**143**:736-9;1989

[167] Kauhanen S., Leivo I., Michelsson J.-E. Early muscle changes after immobilization. *Clin Ortho Relat Res* ;**297**:44-50;1993

[168] Kauhanen S., Leivo I., Pettila M., Michelsson J.-E. Recovery of skeletal muscle after immobilization of rabbit hindlimb. *APMIS* ;**104**:797-804;1996

[169] Kearns S.R., Daly A.F., Sheehan K., Murray P., Kelly C., Bouchier-Hayes D. Oral vitamin C reduces injury to skeletal muscle caused by compartment syndrome. *J Bone Joint Surg (Br)* ;**86-B**:906-11;2004

[170] Kearns S.R., Moneley D., Murray P., Kelly C., Daly A.F. Oral vitamin C attenuates acute ischaemia-reperfusion injury in skeletal muscle. *J Bone Joint Surg (Br)* ;**83**:1202-6;2001

[171] Kilbourn R.G., Gross S.S., Jubran A., Adams J., Griffith O.W., Levi R. et al. NG-Methyl-L-Arginine Inhibits Tumor Necrosis Factor-Induced Hypotension: Implications for the Involvement of Nitric Oxide. *Proc Natl Acad Sci USA* ;**87**:3629-32;1990

[172] Killar L., MacDonald G., West J., Woods A., Bottomly K. Cloned, Ia-restricted T cells that do not produce interleukin 4(IL 4)/B cell stimulatory factor 1(BSF-1) fail to help antigen-specific B cells. *J Immunol* ;**138**:1674-9;1987

[173] Kim J.M., Brannan C.I., Copeland N.G., Jenkins N.A., Khan T.A., Moore K.W. Structure of the mouse IL-10 gene and chromosomal localization of the mouse and human genes. *J Immunol* ;**148**:3618-23;1992

[174] Klenerman L., Lowe N.M., Fryer P.R., Green C.J., Jackson M.J. Dantrolene sodium protects against experimental ischemia and reperfusion damage to skeletal muscle. *Acta Orthop Scand* ;**66**:352-8;1995

[175] Knight K.R., Messina A., Hurley J.V., Zhang B., Morrison W.A., Stewart A.G. Muscle cells become necrotic rather than apoptotic during reperfusion of ischaemic skeletal muscle. *Int J Exp Path* ;**80**:169-75;1999

[176] Kobzik L., Reid M.B., Bredt D.S., Stamler J.S. Nitric oxide in skeletal muscle. *Nature* ;**372**:546-8;1994

[177] Kobzik L., Stringer B., Balligand J.-L., Reid M.B., Stamler J.S. Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. *Biochem Biophys Res Commun* ;**211**:375-81;1995

[178] Koesterer T.J., Dodd S.L., Powers S. Increased antioxidant capacity does not attenuate muscle atrophy caused by unweighting. *J Appl Physiol* ;**93**:1959-65;2002

[179] Koken T., Serteser M., Kahraman A., Akbulut G., Dilek O.N. Which is more effective in the prevention of renal ischemia-reperfusion-induced oxidative injury in the early period in mice: interleukin (IL)-10 or anti-IL-12? *Clin Biochem* ;**37**:50-5;2004

[180] Koksal C., Bozkurt A.K., Cangel U., Ustundag N., Konukoglu D., Musellim B. et al. Attenuation of ischemia/reperfusion injury by *N*-acetylcysteine in a rat hind limb model. *J Surg Res* ;**111**:236-9;2003

[181] Kondo H., Miura M., Itokawa Y. Oxidative stress in skeletal muscle atrophied by immobilization. *Acta Physiol Scand* ;**142**:527-8;1991

[182] Kondo H., Miura M., Nakagaki I., Sasaki S., Itokawa Y. Trace element movement and oxidative stress in skeletal muscle atrophied by immobilization. *Am J Physiol Endocrinol Metab* ;**262**:E583-E590;1992

[183] Kondo H., Nishino K., Itokawa Y. Hydroxyl radical generation in skeletal muscle atrophied by immobilization. *FEBS Lett* ;**349**:169-82;1994

[184] Konishi Y., Aihara Y., Sakai M., Ogawa G., Fukubayashi T. Gamma loop dysfunction in the quadriceps femoris of patients who underwent anterior cruciate ligament reconstruction remains bilaterally. *Scand J Med Sci Sports* ;**17**:393-9;2007

[185] Konishi Y., Fukubayashi T., Takeshita D. Mechanism of quadriceps femoris muscle weakness in patients with anterior cruciate ligament reconstruction. *Scand J Med Sci Sports* ;**12**:371-5;2002

[186] Konishi Y., Fukubayashi T., Takeshita D. Possible mechanism of quadriceeps femoris weakness in patients with ruptured anterior cruciate ligament. *Med Sci Sports Exerc* ;**34**:1414-8;2002

[187] Konishi Y., Ikeda K., Nishino A., Sunaga M., Aihara Y., Fukubayashi T. Relationship between quadriceps femoris muscle volume and muscle torque after anterior cruciate ligament repair. *Scand J Med Sci Sports* ;**17**:656-61;2007

[188] Konishi Y., Konishi H., Fukubayashi T. Gamma loop dysfunction in quadriceps on the contralateral side in patients with ruptured ACL. *Med Sci Sports Exerc* ;**35**:897-900;2003

[189] Korthuis R.J., Carden D.L., Kvietys P.R., Shepro D., Fuseler J. Phalloidin attenuates postischemic neutrophil infiltration aand increasaed microvascular permeability. *J Appl Physiol* ;**71**:1261-9;1991

[190] Korthuis R.J., Granger D.N., Townsley M.I., Tayler A.E. The role of oxygen-derived free radicals in ischemia-induced increases in canine skeletal muscle vascular permeability. *Circ Res* ;**57**:599-609;1985

[191] Kosmidou I., Vassilakopoulos T., Xagorari A., Zakynthino S., Papapetropoulos A., Roussos C. Production of interleukin-6 by skeletal myotubes. *Am J Respir Cell Mol Biol* ;**26**:587-93;2002

[192] Krzyszton C.P., Sparkman N.L., Grant R.W., Buchanan J.B., Broussard S.R., Woods J. et al. Exacerbated fatigue and motor deficits in interleukin-10-deficient mice after peripheral immune stimulation. *Am J Physiol Regul Integr Comp Physiol* ;**295**:R1109-R1114;2008

[193] Labbe R., Lindsay T., Walker P.M. The extent and distribution of skeletal muscle necrosis after grader periods of complete ischemia. *J Vasc Surg* ;**6**:152-7;1987

[194] Lakshminarayanan V., Beno D.W., Costa R.H., Roebuck K.A. Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by H2O2 and tumor necrosis factor-alpha in endothelial and epithelial cells. *J Biol Chem* ;**272**:32910-8;1997

[195] Larsen A.I., Lindal S., Aukrust P., Toft I., Aarsland T., Dickstein K. Effect of exercise training on skeletal muscle fibre characteristics in men with chronic heart failure. Correlation between skeletal muscle alterations, cytokines and exercise capacity. *Int J Cardiol* ;**83**:25-32;2002

[196] Lawler J.M., Song W., Demaree S.R. Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radic Biol Med* ;**35**:9-16;2003

[197] Le Moine O., Louis H., Stordeur P., Collet J.M., Goldman M., Deviere J. Role of reactive oxygen intermediates in interleukin 10 release after cold liver ischemia and reperfusion in mice. *Gastroenterology* ;**113**:1701-6;1997

[198] Le Moine O., Stordeur P., Schandene L., Marchant A., de Groote D., Goldman M. et al. Adenosine enhances IL-10 secretion by human monocytes. *J Immunol* ;**156**:4408-14;1996

[199] Leivo I., Kauhanen S., Michelsson J.-E. Abnormal mitochondria and sarcoplasmic changes in rabbit skeletal muscle induced by immobilization. *APMIS* ;**106**:1113-23;1998

[200] Levine B., Kalman J., Mayer L., Fillit H.M., Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* ;**323**:236-41;1990

[201] Levine M. New concepts in the biology and biochemistry of ascorbic acid. *N Engl J Med* ;**314**:892-902;1986

[202] Levine M., Conry-Cantilena C., Wang Y., Welch R.W., Washko P.W., Dhariwal K.R. et al. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* ;**93**:3704-9;1996

[203] Levine S., Nguyen T., Taylor N., Friscia M.E., Budak M.T., Rothenberg P. et al. Rapid disuse atrophy of diaphragm fibers in mechanically ventilated humans. *N Engl J Med* ;**358**:1327-35;2008

[204] Li G., Labruto F., Sirsjo A., Chen F., Vaage J., Valen G. Myocardial protection by remote preconditioning: the role of nuclear factor kappa-B p105 and inducible nitric oxide synthase. *Eur J Cardiothorac Surg* ;**26**:968-73;2004

[205] Li X., Moody M.R., Engel D., Walker S., Clubb F.J., Sivasubramanian N. et al. Cardiac-specific overexpression of tumor necrosis factor-α causes oxidative stress and contractile dysfunction in mouse diaphragm. *Circulation* ;**102**:1690-6;2000

[206] Li Y.P., Atkins C.M., Sweatt J.D., Reid M.B. Mitochondria mediate tumor necrosis factor-α/NF-κB signaling in skeletal muscle myotubes. *Antioxid Red Signal* ;**1**:97-104;1999

[207] Li Y.P., Chen Y., John J., Moylan J., Jin B., Mann D.L. et al. TNF-α acts via p38 MAPK to stimulate expression of ubiquitin ligase atrogin 1/MAFbx in skeletal muscle. *FASEB J* ;**19**:362-70;2005

[208] Li Y.P., Chen Y., Li A.S., Reid M.B. Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am J Physiol Cell Physiol* ;**285**:C806-C812;2003

[209] Li Y.P., Reid M.B. NF-kB mediates the protein loss induced by TNF-α in differentiated skeletal muscle myotubes. *Am J Physiol Regul Integr Comp Physiol* ;**279**:R1165-R1170;2000

[210] Li Y.P., Reid M.B. NF-κB mediates the protein loss induced by TNF-α in differentiated skeletal muscle myotubes. *Am J Physiol Regul Integr Comp Physiol* ;**279**:R1165-R1170;2000

[211] Li Y.P., Reid M.B. Effect of tumor necrosis factor-α on skeletal muscle metabolism. *Curr Opin Rhem* ;**13**:483-7;2001

[212] Li Y.P., Schwartz R.J., Waddell I.D., Holloway B.R., Reid M.B. Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-κB activation in response to tumor necrosis factor α. *FASEB J* ;**12**:871-80;1998

[213] Li Y.P., Schwartz R.J., Waddell I.D., Holloway B.R., Reid M.B. Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-{kappa}B activation in response to tumor necrosis factor {alpha}. *FASEB J* ;**12**:871-80;1998

[214] Lieber R.L., Pedowitz R.A., Friden J., Gershuni D.H. Decreased muscle speed, strength, and fatigability following two hours of tourniquet-induced ischaemia. *Scand J Plast Reconstr Hand Surg* ;**26**:127-32;1992

[215] Lieber R.L., Silva P.D., Daniel D.M. Equal effectiveness of electrical and volitional strength training for quadriceps femoris muscles after anterior cruciate ligament surgery. *J Orthop Res* ;**14**:131-8;1996

[216] Lindsay T., Walker P.M., Mickle D.A.G., Romaschin A. Measurement of hydroxy-conjugaated dienes aafter ischemia-reperfusion in canin skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**254**:H578-H583;1988

[217] Lindsay T., Walker P.M., Mickle D.A.G., Romaschin A. Measurement of hydroxy-conjugaated dienes aafter ischemia-reperfusion in canine skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**254**:H578-H583;1988

[218] Liu J., Wang X., Mori A. Immobilization stress-induced antioxidant defense changes in rat plasma: effect of treatment with reduced glutathione. *Int J Biochem* ;**26**:511-7;1994

[219] Liu M.J., Li J.X., Lee K.M., Qin L., Chan K.M. Oxidative stress after muscle damage from immobilization and remobilization occurs locally and systemically. *Clin Orthop Relat Res* ;**434**:246-50;2005

[220] Llovera M., Garcia-Martinez C., Agell N., Lopez-Soriano F.J., Argiles J.M. TNF can directly induce the expression of ubiquitin-dependent proteolytic system in rat soleus muscles. *Biochem Biophys Res Commun* ;**230**:238-41;1987

[221] Llovera M., Garcia-Martinez C., Lopez-Soriano J., Agell N., Lopez-Soriano F.J., Garcia I. et al. Protein turnover in skeletal muscle of tumour-bearing transgenic mice overexpressing the soluble TNF receptor-1. *Cancer Letters* ;**130**:19-27;1998

[222] Lopresti C., Kirkendall D.T., Street G.M., Dudley A.W. Quadriceps Insufficiency following Repair of the Anterior Cruciate Ligament*. *J Orthop Sports Phys Ther* ;**9**:245-9;1988

[223] Lorentzon R., Elmqvist L.-G., Sjostrom M., Fagerlund M., Fuglmeyer A.R. Thigh musculature in relation to chronic anterior cruciate ligament tear: Muscle size, morphology, and mechanical output before reconstruction. *Am J Sports Med* ;**17**:423-9;1989

[224] Macatonia S.E., Hosken N.A., Litton M., Vieira P., Hsieh C.S., Culpepper J.A. et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* ;**154**:5071-9;1995

[225] Mallat Z., Heymes C., Ohan J., Faggin E., Leseche G., Tedgui A. Expression of interleukin-10 in advanced human atherosclerotic plaques: relation to inducible nitric oxide synthase expression and cell death. *Arterioscler Thromb Vasc Biol* ;**19**:611-6;1999

[226] Malmberg K.J., Arulampalam V., Ichihara F., Petersson M., Seki K., Andersson T. et al. Inhibition of activated/memory (CD45RO(+)) T cells by oxidative stress associated with block of NF-kappaB activation. *J Immunol* ;**167**:2595-601;2001

[227] Malmberg K.J., Lenkei R., Petersson M., Ohlum T., Ichihara F., Glimelius B. et al. A short-term dietary supplementation of high doses of vitamin E increases T helper 1 cytokine production in patients with advanced colorectal cancer. *Clin Cancer Res* ;**8**:1772-8;2002

[228] Marechal G., Beckers-Bleukx G. Effect of nitric oxide on the maximal velocity of shortening of a mouse skeletal muscle. *Pflugers Arch-Eur J Physiol* ;**436**:906-13;1998

[229] Martins T.B., Anderson J.L., Muhlestein J.B., Horne B.D., Carlquist J.F., Roberts W.L. et al. Risk factor analysis of plasma cytokines in patients with coronary artery disease by a multiplexed fluorescent immunoassay. *Am J Clin Pathol* ;**125**:906-13;2006

[230] Martins T.B., Pasi B.M., Pickering J.W., Jaskowski T.D., Litwin C.M., Hill H.R. Determination of cytokine responses using a multiplexed fluorescent microsphere immunoassay. *Am J Clin Pathol* ;**118**:346-53;2002

[231] Massion P.P., Linden A., Inoue H., Mathy M., Grattan K.M., Nadel J.A. Dimethyl sulfoxide decreases interleukin-8-mediated neutrophil recruitment in the airways. *Am J Physiol Lung Cell Mol Physiol* ;**271**:L838-L843;1996

[232] Mastaloudis A., Morrow J.D., Hopkins D.W., Devaraj S., Traber M.G. Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners. *Free Radic Biol Med* ;**36**:1329-41;2004

[233] Mastaloudis A., Traber M.G., Carstensen K., Widrick J.J. Antioxidant did not prevent muscle damage in response to an ultramarathon run. *Med Sci Sports Exerc* ;**38**:72-80;2006

[234] Matuszczak Y., Arbogast S., Reid M.B. Allopurinol mitigates muscle contractile dysfunction caused by hindlimb unloading in mice. *Aviat Space Environ Med* ;**75**:581-8;2004

[235] McAdam B.F., Mardini I.A., Habib A., Burke A., Lawson J.A., Kapoor S. et al. Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid and isoeicosanoid production in inflammation. *J Clin Invest* ;**105**:1473-82;2000

[236] McArdle A., van der Meulen J., Catapano M., Symons M.C.R., Faulkner J.A., Jackson M.J. Free radical activity following contraction-induced injury to the extensor digitorum longus muscles of rats. *Free Radic Biol Med* ;**26**:1085-91;1999

[237] McClung J.M., kavazis A.N., Whidden M.A., DeRuisseau K.C., Falk D.J., Criswell D.S. et al. Antioxidant Administration Attenuates Mechanical Ventilation-Induced Rat Diaphragm Muscle Atrophy Independent of Protein Kinase B (PKB/Akt) Signaling. *J Physiol (Lond)* ;**585**:203-15;2007

[238] McCutchan H.J., Schwappach J.R., Enquist E.G., Walden D.L., Terada L.S., Reiss O.K. et al. Xanthine oxidase-derived H2O2 contribute to reperfusion injury of ischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**258**:H1415-H1419;1990

[239] McHugh M.P., Tyler T.F., Gleim G.W., Nicholas S.J. Preoperative indicators of motion loss and weakness following anterior cruciate ligament reconstruction. *JOSPT* ;**27**:407-11;1998

[240] McHugh M.P., Tyler T.F., Nicholas S.J., Browne M.G., Gleim G.W. Electromyographic analysis of quadriceps fatigue after anterior cruciate ligament reconstruction. *JOSPT* ;**31**:25-32;2001

[241] McKenna M.J., Medved I., Goodman C.A., Brown M.J., Bjorksten A.R., Murphy K.T. et al. N-acetylcysteine attenuates the decline in muscle Na+,K+-pump activity and delays fatigue during prolonged exercise in humans. *J Physiol (Lond)* ;**576**:279-88;2006

[242] Mecocci P., Fano G., Fulle S., MacGarvey U., Shinobu L., Polidori M.C. et al. Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. *Free Radic Biol Med* ;**26**:303-8;1999

[243] Messina A., Knight K.R., Dowsing B.J., Zhang B., Phan L.H., Hurley J.V. et al. Localization of inducible nitric oxide synthase to mast cells during ischemai/reperfusion injury of skeletal muscle. *Lab Invest* ;**80**:423-31;2000

[244] Meydani S.N., Barklund M.P., Liu S., Meydani M., Miller R.A., Cannon J.G. et al. Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. *Am J Clin Nutr* ;**52**:557-63;1990

[245] Michelsson J.-E., Aho H.J., Kalimo H., Haltia M. Severe degeneration of rabbit vastus intermedius muscle immobilized in shortened position. *APMIS* ;**98**:336-44;1990

[246] Miller R.R., Shardell M.D., Hicks G.E., Cappola A.R., Hawkes W.G., Yu-Yahiro J.A. et al. Association between interleukin-6 and lower extremity function after hip fracture--the role of muscle mass and strength. *J Am Geriatr Soc* ;**56**:1050-6;2008

[247] Mitchell P.O., Pavlath G.K. Skeletal muscle atrophy leads to loss and dysfunction of muscle precursor cells. *Am J Physiol Cell Physiol* ;**287**:C1753-C1762;2004

[248] Miyazawa K., Mori A., Miyata H., Akahane M., Ajisawa Y., Okudaira H. Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J Biol Chem* ;**273**:24832-8;1998

[249] Moopanar T.R., Allen D.G. Reactive oxygen species reduce myofibrillar Ca2+ sensitivity in fatiguing mouse skeletal muscle at 37 degrees C. *J Physiol (Lond)* ;**564**:189-99;2005

[250] Moopanar T.R., Allen D.G. The activity-induced reduction of myofibrillar Ca^{2+} sensitivity in mouse skeletal muscle is reversed by dithiothreitol. *J Physiol (Lond)* ;**571**:191-200;2006

[251] Moore K.W., de Waal M.R., Coffman R.L., O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* ;**19**:683-765;2001

[252] Moore K.W., Vieira P., Fiorentino D.F., Trounstine M.L., Khan T.A., Mosmann T.R. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science* ;**248**:1230-4;1990

[253] Morris C.A., Morris L.D., Kennedy A.R., Sweeney H.L. Attenuation of skeletal muscle atrophy via protease inhibition. *J Appl Physiol* ;**99**:1719-27;2005

[254] Morrison R.J., Miller III C.C., Reid M.B. Nitric oxide effects on shortening velocity and power production in the rat diaphragm. *J Appl Physiol* ;**80**:1065-9;1996

[255] Morrison R.J., Miller III C.C., Reid M.B. Nitric oxide effects of force-velocity characteristics of the rat diaphragm. *Comp Biochem Physiol* ;**119A**:203-9;1998

[256] Morrow J.D., Hill K.E., Burk R.F., Nammour T.M., Badr K.F., Roberts L.J. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci USA* ;**87**:9383-7;1990

[257] Myrianthefs P.M., Lazaris N., Venetsanou K., Smigadis N., Karabatsos E., Anastasiou-Nana M.I. et al. Immune status evaluation of patients with chronic heart failure. *Cytokine* ;**37**:150-4;2007

[258] Nagel J.E., Chopra R.K., Chrest F.J., McCoy M.T., Schneider E.L., Holbrook N.J. et al. Decreased proliferation, interleukin 2 synthesis, and interleukin 2 receptor expression are accompanied by decreased mRNA expression in phytohemagglutinin-stimulated cells from elderly donors. *J Clin Invest* ;**81**:1096-102;1988

[259] Nathens A.B., Neff M.J., Jurkovich G.J., Klotz P., Farver K., Ruzinski J.T. et al. Randomized, prospective trial of antioxidant supplementation in critically ill surgical patients. *Ann Surg* ;**236**:814-22;2002

[260] Nicholas S.J., Tyler T.F., McHugh M.P., Gleim G.W. The effect of leg strength of tourniquet use during anterior cruciate ligament reconstruction: A prospective randomized study. *Arthroscopy* ;**17**:603-7;2001

[261] Nicholls S.J., Hazen S.L. Myeloperoxidase and cardiovascular disease. *Arterioscler Thromb Vasc Biol* ;**25**:1102-11;2005

[262] Nicks D.K., Beneke W.M., Key R.M., Timson B.F. Muscle fiber size and number following immobilisation atrophy. *J Anat* ;**163**:1-5;1989

[263] Novelli G.P., Adembri C., Gandini E., Orlandini S.Z., Papucci L., Formigli L. et al. Vitamin E protects human skeletal muscle from damage during surgical ischemia-reperfusion. *Am J Surg* ;**172**:206-9;1996

[264] Noyes F.R., Mangine R.E., Barber S. Early knee motion after open and arthroscopic anterior cruciate ligament reconstruction. *Am J Sports Med* ;**15**:149-60;1987

[265] O'Neal C.A., Stebbins C.L., Bonigut S., Halliwell B., Longhurst J.C. Production of hydroxyl radicals in contracting skeletal muscle of cats. *J Appl Physiol* ;**81**:1197-206;1996

[266] Ortaldo J.R., Mason A.T., Gerard J.P., Henderson L.E., Farrar W., Hopkins R.F., III et al. Effects of natural and recombinant IL 2 on regulation of IFN gamma production and natural killer activity: lack of involvement of the Tac antigen for these immunoregulatory effects. *J Immunol* ;**133**:779-83;1984

[267] Ostman B., Michaelsson K., Rahme H., Hilered L. Tourniquet-induced ischemia and reperfusion in human skeletal muscle. *Clin Orthop* ;**418**:260-5;2004

[268] Pansarasa O., Bertorelli L., Vecchiet J., Felzani G., Marzatico F. Age-dependent changes of antioxidant activities and markers of free radical damage in human skeletal muscle. *Free Radic Biol Med* ;**27**:617-22;1999

[269] Pansarasa O., Felzano G., Vecchiet J., Marzatico F. Antioxidant pathways in human aged skeltal muscle: relationship with the distribution of type II fibers. *Exp Gerontol* ;**37**:1069-75;2002

[270] Parks D.A., Granger D.N. Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. *Am J Physiol Gas Liv Phy* ;**245**:G285-G289;1983

[271] Patel P., Qi W.-N., Allen D.M., Chen L.-E., Seaber A.V., Stamler J.S. et al. Inhibition of inos with 1400w improves contractile function and alters nos gene and protein expression in reperfused skeletal muscle. *Microsurgery* ;**24**:324-31;2004

[272] Pattwell D., Ashton T., McArdle A., Griffiths R.D., Jackson M.J. Ischemia and reperfusion of skeletal muscle lead to appearance of a stable lipid free radical in the circulation. *Am J Physiol Heart Circ Physiol* ;**284**:H2400-H2404;2003

[273] Pattwell D., McArdle A., Griffiths R.D., Jackson M.J. Measurement of free radical production by in vivo microdiaalysis during ischemia/reperfusion injury to skeletal muscle. *Free Radical Biology & Medicine* ;**30**:979-85;2001

[274] Perkins W.J., Han Y.-S., Sieck G.C. Skeletal muscle force and actomyosin ATPase activity reduced by nitric oxide donor. *J Appl Physiol* ;**83**:1326-32;1997

[275] Peterson J.M., Feeback K.D., Bass J.H., Pizza F.X. Tumor necrosis factor-alpha promotes the accumulation of neutrophils and macrophages in skeletal muscle. *J Appl Physiol* ;**101**:1394-9;2006

[276] Petrasek P.F., Homer-Vanniasinkam S., Walker P.M. Determinants of ischemic injury to skeletal muscle. *Circ Res* ;**19**:623-31;1994

[277] Phan L.H., Hickey M.J., Niazi Z.B.M., Stewart A.G. Nitric oxide synthase inhibitor, nitro-iminoethyl-l-ornithine, reduces ischemia-reperfusion injury in rabbit skeletal muscle. *Microsurgery* ;**15**:703-7;1994

[278] Phillips L., Toledo A.H., Lopez-Neblina F., Anaya-Prado R., Toledo-Pereyra L.H. Nitric oxide mechanism of protection in ischemia and reperfusion injury. *J Invest Surg* ;**22**:46-55;2009

[279] Pierce J.R., Clark B.C., Ploutz-Snyder L.L., Kanaley J.A. Growth hormone and muscle function responses to skeletal muscle ischemia. *J Appl Physiol* ;**101**:1588-95;2006

[280] Plant D.R., Lynch G.S., Williams D.A. Hydrogen peroxide modulates Ca2+-activation of single permeabilized fibres from fast- and slow-twitch skeletal muscles of rats. *J Muscle Res Cell Motil* ;**21**:747-52;2000

[281] Plant D.R., Lynch G.S., Williams D.A. Hydrogen peroxide increases depolarization-induced contraction of mechanically skinned slow twitch fibres from rat skeletal muscles. *J Physiol (Lond)* ;**539**:883-91;2002

[282] Plomgaard P., Penkowa M., Pedersen B.K. Fiber type specific expression of TNF-alpha, IL-6 and IL-18 in human skeletal muscles. *Exer Immunol Rev* ;**11**:53-63;2005

[283] Podda M., Weber C., Traber M.G., Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones. *J Lipid Res* ;**37**:893-901;1996

[284] Podolin R.A., Ford L.E. Influence of partial activation on force-velocity properties of frog skinned muscle fibers in millimolar magnesium ion. *J Gen Physiol* ;**87**:607-31;1986

[285] Posterino G.S., Lamb G.D. Effects of reducing agents and oxidatns on excitation-contraction coupling in skeletal muscle fibres of rat and toad. *The Journal of Physiology (London)* ;**496**:809-25;1996

[286] Powers S.K., Jackson M.J. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev* ;**88**:1243-76;2008

[287] Powers S.K., kavazis A.N., McClung J.M. Oxidative stress and disuse muscle atrophy. *J Appl Physiol* ;**102**:2389-97;2007

[288] Proske U., Morgan D.L. Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. *J Physiol (London)* ;**537**:333-45;2001

[289] Qi W.-N., Chen L.-E., Seaber A.V., Urbaniak J.R. Regulation of NOS mRNA expression in reperfused muscle. *Microsurgery* ;**19**:18-9;1999

[290] Qi W.-N., Zhang L., Chen L.-E., Seaber A.V., Urbaniak J.R. Nitric oxide involvement in reperfusion injury of denervated muscle. *J Hand Surg* ;**29A**:638-45;2004

[291] Qi W.N., Chen L.E., Zhang L., Eu J.P., Seaber A.V., Urbaniak J.R. Reperfusion injury in skeletal muscle is reduced in inducible nitric oxide synthase knockout mice. *J Appl Physiol* ;**97**:1323-8;2004

[292] Qi W.N., Yan Z.Q., Whang P.G., Zhou Q., Chen L.E., Seaber A.V. et al. Gene and protein expressions of nitric oxide synthases in ischemia-reperfused peripheral nerve of the rat. *Am J Physiol Cell Physiol* ;**281**:C849-C856;2001

[293] Qiao H., Bell J., Juliao S., Li L., May J.M. Ascorbic acid uptake and regulation of type I collagen synthesis in cultured vascular smooth muscle cells. *J Vasc Res* ;**46**:15-24;2009

[294] Racz I.B., Illyes G., Sarkadi L., Hamar J. The functional and morphological damage of ischemic reperfused skeletal muscle. *Eur Surg Res* ;**29**:254-63;1997

[295] Racz I.B., Sarkadi L., Hamar J. The functional damages of ischemic/reperfused skeletal muscle. *Acta Physiol Hungaria* ;**84**:205-16;1996

[296] Reid M.B. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand* ;**162**:401-9;1998

[297] Reid M.B. Free radicals and muscle fatigue: Of ROS, canaries, and the IOC. *Free Radic Biol Med* ;**44**:169-79;2008

[298] Reid M.B., Andrade F.H., Balke C.W., Esser K.A. Redox mechanisms of muscle dysfunction in inflammatory disease. *Phys Med Rehabil Clin N Am* ;**16**:925-49;2005

[299] Reid M.B., Haack K.E., Franchek K.M., Valber P.A., Kobzik L., West M.S. Reactive oxygen in skeletal muscle I. intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol* ;**73**:1797-804;1992

[300] Reid M.B., Kobzik L., Bredt D.S., Stamler J.S. Nitric oxide modulates excitation-contraction coupling in the diaphragm. *Comp Biochem Physiol* ;**119A**:211-8;1998

[301] Reid M.B., Lannergren J., Westerblad H. Respiratory and limb muscle weakness induced by tumor necrosis factor-α. *Am J Respir Crit Care Med* ;**166**:479-84;2002

[302] Reid M.B., Moody M.R. Dimethyl sulfoxide depresses skeletal muscle contractility. *J Appl Physiol* ;**76**:2186-90;1994

[303] Reid M.B., Stokic D.S., Koch S.M., Khawli F.A., Leis A.A. N-acetylcysteine inhibits muscle fatigue in humans. *J Clin Invest* ;**94**:2468-74;1994

[304] Rennie M.J., Atherton P., Selby A., Smith K., Narici M., de Boer M. et al. Letter to the Editor on the Journal Club article by Barker and Traber. *J Physiol (Lond)* ;**586**:307-8;2008

[305] Riede U.N., Forstermann U., Drexler H. Inducible nitric oxide synthase in skeletal muscle of patients with chronic heart failure. *J Am Coll Cardiol* ;**32**:964-9;1998

[306] Rink L., Cakman I., Kirchner H. Altered cytokine production in the elderly. *Mech Ageing Dev* ;**102**:199-209;1998

[307] Risberg M.A., Holm I., Steen H., Eriksson J., Ekeland A. The Effect of Knee Bracing After Anterior Cruciate Ligament Reconstruction: A Prospective, Randomized Study with Two Years' Follow-up. *Am J Sports Med* ;**27**:76-83;1999

[308] Roberts L.J., Oates J.A., Linton M.F., Fazio S., Meador B.P., Gross M.D. et al. The relationship between dose of vitamin E and suppression of oxidative stress in humans. *Free Radic Biol Med* ;**43**:1388-93;2007

[309] Rosenberg T.D., Franklin J.L., Baldwin G.N., Nelson K.A. Extensor mechanism function after petallar tendon graft harvest for anterior cruciate ligament reconstruction. *Am J Sports Med* ;**20**:519-26;1992

[310] Rubin B.B., Chang G., Liauw S., Young A., Romaschin A., Walker P.M. Phospholipid peroxidation deacylation and remodeling in postischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**263**:H1695-H1702;1992

[311] Rubin B.B., Liauw S., Tittley J., Romaschin A., Walker P.M. Prolonged adenine nucleotide resynthesis and reperfusion injury in postischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**262**:H1538-H1547;1992

[312] Rubin B.B., Romaschin A., Walker P., Walker P.M., Gute D.C., Korthuis R.J. Mechanisms of postischemic injury in skeletal muscle: intervention strategies. *J Appl Physiol* ;**80**:369-87;1996

[313] Rubin B.B., Smith A., Liauw S., Isenman D., Romaschin A.D., Walker P.M. Complement activation and white cell sequestration in postischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**259**:H525-H531;1990

[314] Rubin R., Tittley J.T., Chang G., Smith A., Liauw S., Romaschin A. et al. A clinically applicable method for long-term salvage of postischemic skeletal muscle. *J Vasc Surg* ;**13**:58-68;1991

[315] Rudnick J., Puttmann B., Tesch P.A., Alkner B., Schoser B.G.H., Salanova M. et al. Differential expression of nitric oxide synthases (NOS 1-3) in human skeletal muscle following exercise countermeasure during 12 weeks of bed rest. *FASEB J* ;**18**:1228-30;2004

[316] Ryall J.G., Schertzer J.D., Lynch G.S. Cellular and molecular mechanisms underlying age-related skeletal muscle wasting and weakness. *Biogerontology* ;**9**:213-28;2008

[317] Salonen R.M., Nyyssonen K., Kaikkonen J., Porkkala-Sarataho E., Voutilainen S., Rissanen T.H. et al. Six-year effect of combined vitamin C and E supplementation on atherosclerotic progression: the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study. *Circulation* ;**107**:947-53;2003

[318] Sander M., Irwin M., Sinha P., Naumann E., Kox W.J., Spies C.D. Suppression of interleukin-6 to interleukin-10 ratio in chronic alcoholics: association with postoperative infections. *Intensive Care Med* ;**28**:285-92;2002

[319] Saricaoglu F., Dal D., Salman A.E., Atay O.A., Doral M.N., Salman M.A. et al. Effect of low-dose n-acetyl-cysteine infusion on tourniquet-induced ischaemia-reperfusion injury in arthroscopic knee surgery. *Acta Anaesthesiol Scand* ;**49**:847-51;2005

[320] Schaap L.A., Pluijm S.M., Deeg D.J., Visser M. Inflammatory markers and loss of muscle mass (sarcopenia) and strength. *Am J Med* ;**119**:526-17;2006

[321] Schlag M.G., Clarke S., Carson M.W., Harris K.A., Potter R.F. The effect of mannitol versus dimethyl thiourea at attenuating ischemia/reperfusion-induced injury to skeletal muscle. *J Vasc Surg* ;**29**:511-21;1999

[322] Schulze-Osthoff K., Bakker A.C., Vanhaesebroeck B., Beyaert R., Jacob W.A., Fiers W. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J Biol Chem* ;**267**:5317-23;1992

[323] Schulze-Osthoff K., Beyaert R., Vandevoorde V., Haegeman G., Fiers W. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J* ;**12**:3095-104;1993

[324] Seekamp A., Mulligan M.S., Till G.O., Ward P.A. Requirments for neutrophil products and L-arginine in ischemia-reperfusion injury. *Am J Pathol* ;**142**:1217-26;1993

[325] Selsby J.T., Dodd S.L. Heat treatment reduces oxidative stress and protects muscle mass during immobilization. *Am J Physiol Regul Integr Comp Physiol* ;**289**:R134-R139;2005

[326] Selsby J.T., Rother S., Tsuda S., Pracash O., Quindry J.C., Dodd S.L. Intermittent hyperthermia enhances skeletal muscle regrowth and attenuates oxidative damage following reloading. *J Appl Physiol* ;**102**:1702-7;2006

[327] Sen C.K., Marin E., Kretzschmar M., Hanninen O. Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J Appl Physiol* ;**73**:1265-72;1992

[328] Servais S., Letexier D., Favier R., Duchamp C., Desplanches D. Prevention of unloading-induced atrophy by vitamin E supplementation: links between oxidative stress and soleus muscle proteolysis? *Free Radic Biol Med* ;**42**:627-35;2007

[329] Shah S.B., Peters D., Jordan K.A., Milner D.J., Friden J., Capetanaki Y. et al. Sarcomere number regulation maintained after immobilization in desmin-null mouse skeletal muscle. *J Exp Biol* ;**204**:1703-10;2001

[330] Shi S., Klotz U. Clinical use and pharmacological properties of selective COX-2 inhibitors. *Eur J Clin Pharmacol* ;**64**:233-52;2008

[331] Sies H., Stahl W. Vitamins E and C, β-carotene, and other carotenoids as antioxidants. *Am J Clin Nutr* ;**62S**:1315S-21S;1995

[332] Sindermann J., Kruse A., Frercks H.J., Schutz R.M., Kirchner H. Investigations of the lymphokine system in elderly individuals. *Mech Ageing Dev* ;**70**:149-59;1993

[333] Sirsjo A., Astrand K., Kagedal B., Nylander G., Gidlof A. In situ microdialysis for monitoring of extracellular glutathione levels in normal, ischemia and post-ischemic skeletal muscle. *Free Rad Res* ;**25**:385-91;1996

[334] Sirsjo A., Kagedal B., Arstrand K., Lewis D.H., Nylander G., Gidlog A. Altered glutathione levels in ischemic and postichemic skeletal muscle: difference between severe and moderate ischemic insult. *J Trauma* ;**41**:123-8;1996

[335] Sirsjo A., Lewis D.H., Nylander G. The accumulation of polymorphnuclear leukocytes in post-ischemic skeletal muscle in the rat, measured by quantiting tissue myeloperoxidase. *Int J Microcirc Clin Exp* ;**9**:163-73;1990

[336] Smith J.K., Carden D.L., Grisham M.B., Granger D.N., Korthuis R.J. Role of iron on postischemic microvascular injury. *Am J Physiol Heart Circ Physiol* ;**256**:H1472-H1477;1989

[337] Smith J.K., Grisham M.B., Granger D.N., Korthuis R.J. Free radical defense mechanisms and neutorphil infiltration in postischemic skeletal muscle. *Am J Physiol Heart Circ* ;**256**:H789-H793;1989

[338] Smith K.J., Carden D.L., Korthuis R.J. Role of xanthine oxidase in postischemic microovascula injury in skeletal muscle. *Am J Physiol Heart Circ Physiol* ;**257**:H1782-H1789;1989

[339] Smith L.L., Anwar A., Fragen M., Rananto C., Johnson R., Holbert D. Cytokines and cell adhesion molecules associated with high-intensity eccentric exercise. *Eur J Appl Physiol* ;**82**:61-7;2000

[340] Snyder-Mackler L., Binder-MaCleod S.A., Williams P.R. Fatigability of human quadriceps femoris muscle following anterior cruciate ligament reconstruction. *Med Sci Sports Exerc* ;**25**:783-9;1993

[341] Snyder-Mackler L., De Luca P.F., Williams P.R., Eastlack M.E., Bartolozzi A.R. Reflex inhibition of the quadriceps femoris muscle after injury or reconstruction of the anterior cruciate ligament. *J Bone Joint Surg (Am)* ;**76**:555-60;1994

[342] Soussi B., Lagerwall K., Idstrom J.-P., Schersten T. Purine metabolic pathways in rat hindlimb perfusion model during ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* ;**265**:H1074-H1081;1993

[343] Stamler J.S., Meissner G. Physiology of nitric oxide in skeletal muscle. *Physiol Rev* ;**81**:209-37;2001

[344] Sternbergh III W.C., Adelman B. Skeletal muscle fiber type does not predict sensitivity of postischemic damage. *J Surg Res* ;**53**:535-41;1992

[345] Stout R.D., Bottomly K. Antigen-specific activation of effector macrophages by IFN-gamma producing (TH1) T cell clones. Failure of IL-4-producing (TH2) T cell clones to activate effector function in macrophages. *J Immunol* ;**142**:760-5;1989

[346] Strieter R.M., Kunkel S.L., Showell H.J., Remick D.G., Phan S.H., Ward P.A. et al. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1 beta. *Science* ;**243**:1467-9;1989

[347] Supinski G., Nethery D., DiMarco A. Effect of free radical scavengers on endotoxin-induced respiratory muscle dysfunction. *Am Rev Respir Dis* ;**148**:1318-24;1993

[348] Supinski G.S., Stofan D., Callahan A., Nethery D., Nosek T.M., DiMarco A. Peroxynitrate induces contractile dysfunction and lipid peroxidation in the diaphragm. *J Appl Physiol* ;**87**:783-91;1999

[349] Suzuki H., Poole D.C., Zwelfach B.W., Schmid-Schonbein G.W. Temporal correlation between maximum tetanic force and cell death in postischemic rat skeletal muscle. *J Clin Invest* ;**96**:2892-7;1995

[350] Suzuki N., Motohashi N., Uezumi A., Fukada S., Yoshimura T., Itoyama Y. et al. NO production results in suspension-induced muscle atrophy through dislocation of neuronal NOS. *J Clin Invest* ;**117**:2468-76;2007

[351] Taga K., Tosato G. IL-10 inhibits human T cell proliferation and IL-2 production. *J Immunol* ;**148**:1143-8;1992

[352] Takano H., Manchikalapudi S., Tang X.L., Qiu Y., Rizvi A., Jadoon A.K. et al. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* ;**98**:441-9;1998

[353] Taniguchi T., Koido Y., Aiboshi J., Yamashita T., Suzaki S., Kurokawa A. Change in the ratio of interleukin-6 to interleukin-10 predicts a poor outcome in patients with systemic inflammatory response syndrome. *Crit Care Med* ;**27**:1262-4;1999

[354] Taniguchi T., Koido Y., Aiboshi J., Yamashita T., Suzaki S., Kurokawa A. The ratio of interleukin-6 to interleukin-10 correlates with severity in patients with chest and abdominal trauma. *Am J Emerg Med* ;**17**:548-51;1999

[355] Taylor A.W., Bruno R.S., Frei B., Traber M.G. Benefits of prolonged gradient separation for HPLC-MS-MS quantitation of plasma total 15-Series F2-isoprostanes. *Analytical Biochemistry* ;**350**:41-5;2006

[356] Thom J.M., Thompson M.W., Ruell P.A., Bryant G.J., Fonda J.S., Harmer A.R. et al. Effect of 10-day cast immobilization on sarcoplasmic reticulum calcium regulation in humans. *Acta Physiol Scand* ;**172**:141-7;2001

[357] Thomason D.B., Biggs R.B., Booth F.W. Protein metabolism and beta-myosin heavy-chain mRNA in unweighted soleus muscle. *Am J Physiol Regul Integr Comp Physiol* ;**257**:R300-R305;1989

[358] Tibone J.E., Antich T.J. A biomechanical analysis of anterior cruciate ligament reconstructive with the petallar tendon. A two year followup. *Am J Sports Med* ;**16**:332-5;1988

[359] Tilg H., Atkins M.B., Dinarello C.A., Mier J.W. Induction of circulating interleukin 10 by interleukin 1 and interleukin 2, but not interleukin 6 immunotherapy. *Cytokine* ;**7**:734-9;1995

[360] Tkacova R., Kluchova Z., Joppa P., Petrasova D., Molcanyiova A. Systemic inflammation and systemic oxidative stress in patients with acute exacerbations of COPD. *Respir Med* ;**101**:1670-6;2007

[361] Toft A.D., Jensen L.B., Bruunsgaard H., Ibfelt T., Halkjaer-Kristensen J., Febbraio M.A. et al. Cytokine response to eccentric exercise in young and elderly humans. *Am J Physiol Cell Physiol* ;**283**:C289-C295;2002

[362] Toth M.J., Ades P.A., Tischler M.D., Tracy R.P., LeWinter M.M. Immune activation is associated with reduced skeletal muscle mass and physical function in chronic heart failure. *Int J Cardiol* ;**109**:179-87;2006

[363] Traber M.G., Atkinson J. Vitamin E, antioxidant and nothing more. *Free Radic Biol Med* ;**43**:4-15;2007

[364] Travaline J.M., Sudarshan S., Roy B.G., Cordova F., Leyenson V., Criner G.I. Effect of N-acetylcysteine on human diaphragm strength and fatigability. *Am J Respir Crit Care Med* ;**156**:1567-71;1997

[365] Trinchieri G., Matsumoto-Kobayashi M., Clark S.C., Seehra J., London L., Perussia B. Response of resting human peripheral blood natural killer cells to interleukin 2. *J Exp Med* ;**160**:1147-69;1984

[366] Tsujinaka T., Fujita J., Ebisui C., Yano M., Kominami E., Suzuki K. et al. Interleukin 6áReceptor Antibody Inhibits Muscle Atrophy and Modulates Proteolytic Systems in Interleukin 6áTransgenic Mice. *J Clin Invest* ;**97**:244-9;1996

[367] Tupling R., Green H., Senisterra G., Lepock J., McKee N. Effects of ischemia on sarcoplasmic reticulum Ca2+ uptake and Ca2+ release in rat skeletal muscle. *Am J Physiol Endocrinol Metab* ;**281**:E224-E232;2001

[368] Tyler T.F., McHugh M.P., Gleim G.W., Nicholas S.J. The effect of immediate weightbearing after anterior cruciate ligament reconstruction. *Clin Orthop Relat Res* ;**357**:141-8;1998

[369] Urbach D., Nebelung W., Weiler H.-T., Awiszus F. Bilateral deficit of voluntary quadriceps muscle activation after unilateral ACL tear. *Med Sci Sports Exerc* ;**31**:1691-6;1999

[370] Urso M.L., Scrimgeour A.G., Chen Y.W., Thompson P.D., Clarkson P.M. Analysis of human skeletal muscle after 48 h immobilization reveals alterations in mRNA and protein for extracellular matrix components. *J Appl Physiol* ;**101**:1136-48;2006

[371] van Dissel J.T., van Langevelde P., Westendorp R.G., Kwappenberg K., Frolich M. Anti-inflammatory cytokine profile and mortality in febrile patients. *Lancet* ;**351**:950-3;1998

[372] van Helvoort H.A., Heijdra Y.F., de Boer R.C., Swinkels A., Thijs H.M., Dekhuijzen P.N. Six-minute walking-induced systemic inflammation and oxidative stress in muscle-wasted COPD patients. *Chest* ;**131**:439-45;2007

[373] Visser M., Pahor M., Taaffe D.R., Goodpaster B.H., Simonsick E.M., Newman A.B. et al. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. *J Gerontol A Biol Sci Med Sci* ;**57**:M326-M332;2002

[374] Walden D.L., McCutchan H.J., Enquist E.G., Schwappach J.R., Shanley P.F., Reiss O.K. et al. Neutrophils accumulate and contribute to skeletal muscle

dysfunction after ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* ;**259**:H1809-H1812;1990

[375] Waldmann T.A. The multi-subunit interleukin-2 receptor. *Annu Rev Biochem* ;**58**:875-911;1989

[376] Waldmann T.A., Goldman C.K., Robb R.J., Depper J.M., Leonard W.J., Sharrow S.O. et al. Expression of interleukin 2 receptors on activated human B cells. *J Exp Med* ;**160**:1450-66;1984

[377] Walker P.M., Lindsay T.F., Labbe R., Mickle D.A., Romaschin A.D. Salvage of skeletal muscle with free radical scavengers. *J Vasc Surg* ;**5**:68-75;1987

[378] Wang P., Wu P., Anthes J.C., Siegel M.I., Egan R.W., Billah M.M. Interleukin-10 inhibits interleukin-8 production in human neutrophils. *Blood* ;**83**:2678-83;1994

[379] Wang P., Wu P., Siegel M.I., Egan R.W., Billah M.M. IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. *J Immunol* ;**153**:811-6;1994

[380] Wang Y., Huang D.S., Wood S., Watson R.R. Modulation of immune function and cytokine production by various levels of vitamin E supplementation during murine AIDS. *Immunopharmacology* ;**29**:225-33;1995

[381] Wanidworanun C., Strober W. Predominant role of tumor necrosis factor-alpha in human monocyte IL-10 synthesis. *J Immunol* ;**151**:6853-61;1993

[382] Wigerstad-Lossing I., Grimby G., Jonsson T., Peterson B.M.L., Renstrom P. Effects of electrical stimulation combined with voluntary contractions after knee ligament surgery. *Med Sci Sports Exerc* ;**20**:93-8;1988

[383] Wilcox P., Milliken C., Bressler B. High-dose tumor necrosis factor α produces an impairment of hamster disphragm contractility: Attenuation with a prostaglandin inhibitor. *Am J Respir Crit Care Med* ;**153**:1611-5;1996

[384] Wilcox P.G., Wakai Y., Walley K.R., Cooper D.J., Road J. Tumor necrosis factor α decreases *in vivo* diaphragm contractility in dogs. *Am J Respir Crit Care Med* ;**150**:1368-73;1994

[385] Wilk K.E., Reinold M.M., Hooks T.R. Recent advances in the rehabilitation of isolated and combined anterior cruciate ligament injuries. *Orthop Clin North Am* ;**34**:107-34;2003

[386] Williams G., Brown T., Becker L., Prager M., Giroir B.P. Cytokine-induced expression of nitric oxide synthase in C2C12 skeletal muscle myocytes. *Am J Physiol Regul Integr Comp Physiol* ;**36**:R1020-R1025;1994

[387] Williams G.N., Snyder-Mackler L., Barrance P.J., Buchanan T.S. Quadriceps femoris muscle morphology and function after ACL injury: a differential response in copers versus non-copers. *J Biomech* ;**38**:685-93;2005

[388] Willy C., Dahouk S., Starck C., Kaffenberger W., Gerngross H., Plappert U.G. DNA damage in human leukocytes after ischemia/reperfusion injury. *Free Radic Biol Med* ;**28**:1-12;2000

[389] Wintergerst E.S., Maggini S., Hornig D.H. Contribution of selected vitamins and trace elements to immune function. *Ann Nutr Metab* ;**51**:301-23;2007

[390] Witzmann F.A., Kim D.H., Fitts R.H. Hindlimb immobilization: length-tension and conctractile properties of skeletal muscle. *J Appl Physiol* ;**53**:335-45;1982

[391] Witzmann F.A., Kim D.H., Fitts R.H. Effect of hindlimb immobilization on fatigability of skeletal muscle. *J Appl Physiol* ;**54**:1242-8;1983

[392] Woitaske M.D., McCarter R.J.M. Effects of fiber type on ischemia-reperfusion injury in mouse skeletal muscle. *Plast Reconstr Surg* ;**102**:2052-63;1998

[393] Wu D., Meydani S.N. Age-associated changes in immune and inflammatory responses: impact of vitamin E intervention. *J Leukoc Biol* ;**84**:900-14;2008

[394] Wu J.H., Ward N.C., Indrawan A.P., Almeida C.A., Hodgson J.M., Proudfoot J.M. et al. Effects of alpha-tocopherol and mixed tocopherol supplementation on markers of oxidative stress and inflammation in type 2 diabetes. *Clin Chem* ;**53**:511-9;2007

[395] Xi L., Jarrett N.C., Hess M.L., Kukreja R.C. Essential role of inducible nitric oxide synthase in monophosphoryl lipid A-induced late cardioprotection: evidence from pharmacological inhibition and gene knockout mice. *Circulation* ;**99**:2157-63;1999

[396] Yang Z., Zingarelli B., Szabo C. Crucial role of endogenous interleukin-10 production in myocardial ischemia/reperfusion injury. *Circulation* ;**101**:1019-26;2000

[397] Yokoto J., Minei J.P., Fantini G.A., Shires G.T. Role of leukocytes in reperfusion injury of skeletal muscle after partial ischemia. *Am J Physiol Heart Circ Physiol* ;**257**:H1068-H1075;1989

[398] Young I.S., Trimble E.R. Measurement of malondialdehyde in plasma by high performance liquid chromatography with fluorimetric detection. *Ann Clin Biochem* ;**28 (Pt 5)**:504-8;1991

[399] Zarzhevsky N., Menashe O., Carmeli E., Stein H., Reznick A.Z. Capacity for recovery and possible mechanisms in immobilization atrophy of young and old animals. *Ann N Y Acad Sci* ;**928**:212-25;2001

[400] Zhang L., Looney C.G., Qi W.-N., Chen L.-E., Seaber A.V., Stamler J.S. et al. Reperfusion injury is reduced in skeletal muscle by inhibition of inducible nitric oxide synthase. *J Appl Physiol* ;**94**:1473-8;2003

[401] Zysk S.P., Fraunberger P., Veihelmann A., Dorger M., Kalteis T., Maier M. et al. Tunnel enlargement and changes in synovial fluid cytokine profile following anterior cruciatae ligament reconstruction with petallar tendon and hamstring tendon autografts. *Knee Surg Sports Traumatol Arthrosc* ;**12**:98-103;2004