ISOSORBIDE DINITRATE AND L-ARGININE: FOUNTAIN OF YOUTH FOR AGED MUSCLE REGENERATION?

By

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To my parents, to whom I owe everything

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CL	Control
CTX	Cardiotoxin
H&E	Hematoxylin and eosin
HGF	Hepatic growth factor
ISDN	Isosorbide dinitrate
L-NAME	N-nitro-l-arginine methyl ester
MDX	Mouse muscular dystrophy model
MMP	Matrix metalloproteinase
MRF	Muscle regulatory factor
MYF5	Myogenic factor five
MYOD	Myogenic differentiation antigen
NO	Nitric oxide
NNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
OA	Old l-arginine supplemented mice
OC	Old control supplemented mice
OCT	Optimal cutting temperature compound
OI	Old isorbide dintrate supplemented mice
OPN	Osteopontin
RI	Regeneration index
TA	Tibialis anterior
TACXA	Tibialis anterior cross sectional area
TGF-B	Tumor growth factor beta

Abstract of Thesis Presented to the Graduate School
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Senescent myofibers generally are characterized by a regenerative deficit in comparison to their younger counterparts, one of many symptoms of sarcopenia. This deficit is largely due to the blunting of satellite cell activation, likely caused by systemic factors. In response to injury, nitric oxide is produced and is one of the primary mediators of the subsequent regenerative response. This response entails activation of matrix metalloproteinases, endopeptidases that cleave hepatic growth factor from its extracellular binding site and result in satellite cell activation. It has been well established that L-arginine and isosorbide dinitrate are both potent precursors to NO, and the purpose of our investigation was to determine if either NO donor, enzymatic or non-enzymatic respectively, had any impact in rescuing the myogenic capacity of the senescent muscle. We hypothesized that both supplements would have a positive effect on the regeneration of the treated aged subjects compared to their senescent control counterparts. Each subgroup of mice consisted of four 23-month-old individuals, with four young controls to serve as an index of myogenic rescue. The animals were supplemented with the treatment diets for a week prior to cardiotoxin injection, then sacrificed at either five or ten days post injection. Histochemical analysis was used to

determine extent of regeneration. Our results indicated a recovery of early regenerative capacity with both supplements at five days, but not at ten days. We conclude that while promising, further investigation is necessary to determine the true efficacy of these supplements for the treatment and prevention of sarcopenia.

CHAPTER 1
INTRODUCTION

Background

The importance of understanding the decline of the muscular system as we age is of fundamental importance to human health and wellness. The process of aging in humans has a multitude of effects on the body. However, barring overt disease, the loss of muscle strength and mass with advancing age, termed sarcopenia, and decreased muscular regenerative ability has the greatest impact on quality of life, producing the classic senescent phenotype often referred to as frailty. Sarcopenia and reduced regenerative potential interact to create a “snowball effect” that only amplifies itself as time passes. Nitric oxide (NO), a molecule with seemingly unbounded physiological influence, has been implicated in possible attenuation of these processes due to its involvement in several muscle regeneration pathways, including satellite cell activation, proliferation, and protein synthesis. It is generally accepted that muscle trauma and recovery occurs in three phases: degeneration, regeneration, and reconstruction. NO has been shown to exert some level of control over each of these three phases (17), and may be altered with aging. Therefore, augmentation of NO production or bioavailability may provide a convenient target for pharmacological or nutritional amelioration of age-related deficits in the muscle regeneration process.

Muscle regeneration is a complex process of vital importance in mammalian organisms, controlled through a myriad of pathways. Of utmost significance to this process is the pathway of satellite cell activation and proliferation. Satellite cells are progenitor cells located on the muscle fiber that are quiescent until a sufficient stimulus is received to initiate their activation. It is at this crucial juncture that the satellite cells

undergo proliferation and myogenic differentiation, completing the transition to mature muscle cells. This differentiation is largely controlled by two myogenic regulatory factors, MyoD and Myf5. MyoD has been implicated in the progression to complete differentiation, whereas Myf5 is thought to be involved with renewal of the satellite cell pool (9). It is well established that satellite cell populations in aging muscle are resistant to activation and proliferation, and this is thought to be a primary cause of reduced regenerative potential in aging muscle. This resistance to activation and proliferation was thought to be an intrinsic property of the aging satellite cell until research from Rando and colleagues over the last decade proved otherwise. It is now generally accepted that aging muscle retains a robust regenerative potential if the resident satellite cells are exposed to the proper environment. Specifically, exposure of satellite cells from aging mouse muscle to serum from young mice, either *in vitro* or *in vivo*, restores satellite cell activity in injured muscle and functional recovery of the muscle (12). Ultimately an understanding of the positive and negative regulators of satellite cell activation and proliferation could lead to nutritional or pharmacological methods of rescuing the regenerative potential of aging muscle.

Nitric oxide is a near omnipresent molecule in the human body, as almost every cell possessing a nucleus contains one of the three NOS isoforms. In addition, it performs a variety of functions such as neural signal conduction, vasodilation, and sanitation (5). Recent evidence supports the idea that nitric oxide also exerts regulatory control over muscle regeneration. It has been shown to have a role in every phase of muscle response to injury and exudes control over an immense cascade of reactions, many of which are yet to be discovered. The activation of satellite cells is one of the first

and most important responses to muscle injury, and is initiated by hepatocyte growth factor (HGF) binding to the c-met receptor on quiescent satellite cells. It is now known that nitric oxide controls this process. Muscular NO production is induced through a mechanical stretch stimulus which activates nitric oxide synthase (NOS) and proceeds to induce activation of matrix metalloproteinases (MMPs). MMPs have been shown to proteolyticly release HGF from an extracellular tether, allowing it to quickly activate neighboring satellite cells.

The necessity of NO production for skeletal muscle regeneration was shown by Filippin et al. (2011) who demonstrated that inhibition of NOS activity prevents normal regeneration of skeletal muscle following crush injury in young mice. It has recently been shown that supplemental nitric oxide in vitro, via addition of a nitric oxide donor, can augment satellite cell activation on mechanically stimulated isolated myofibers from old mice (17). Therefore, it is reasonable to hypothesize that supplementation of endogenous NO production in vivo may overcome the aging deficit in satellite cell activity following muscle injury.

L-arginine is an amino acid that serves a plethora of roles within the body. It is a primary intermediate in the Urea cycle and is a major constituent in the production of polyamines as well as glutamate and creatine (24). However, quite possibly its most important physiological role relative to skeletal muscle regeneration is that of precursor to NO production. Through the action of the enzyme NOS, L-arginine is converted to NO and citrulline. L-arginine supplementation has been shown to augment wound healing, presumably via NO-related stimulation of angiogenesis. Only one study has examined L-arginine and skeletal muscle regeneration, reporting that L-arginine

supplementation significantly improved regeneration in a mouse model involving damage to both skeletal muscle fibers and muscle vasculature (25). It is unknown whether this effect was due to NO action on satellite cells or angiogenesis.

Isosorbide Dinitrate (ISDN) is a known vasodilatory agent used in the treatment of heart disease, as well as an NO donor. ISDN has been shown to enhance skeletal muscle regeneration in a mouse model of muscular dystrophy. ISDN has a high potential for clinical efficacy because of a general lack of side effects (22). Further, it does not rely on the action of the NOS enzyme to increase NO availability and should, therefore, retain its therapeutic value even in situations where the NOS enzyme is down-regulated.

Aging skeletal muscle exhibits a significant impairment of regenerative potential, due primarily to inhibition of satellite cell activity by extrinsic factors in the aging environment. The rescue of regenerative potential in aging muscle by exposure of the muscle to serum from a young animal suggests the possibility of successful pharmacological or nutritional strategies to stimulate regeneration in aging muscle. Nitric oxide is a key factor in the activation of satellite cells following muscle injury, and exposure of isolated myofibers from old mice to L-arginine or an NO donor ameliorates the aging deficit in mechanical activation of satellite cells (4). Therefore, the following aims and hypotheses were addressed in this study.

Specific Aims and Hypotheses

Specific Aim: To investigate whether elevated levels of nitric oxide achieved through exogenous supplementation will have positive effects on muscle regeneration in aged mice.

Sub Aim 1: To determine if administration of ISDN will elicit positive changes in muscle regeneration in aged subjects.

Rationale: ISDN is a chemical compound that is widely used in the field of cardiology as a vasodilatory agent, causing the vasculature to expand and increase blood flow. These properties can be attributed to this compound as a result of its status as an NO donor, and it has been shown to improve muscle regeneration in muscular dystrophic mice.

Hypothesis 1: Exogenous supplementation of ISDN will result in a greater extent of muscle regeneration response to injury in aging subjects (22).

Sub Aim 2: To determine if augmented amounts of L-arginine will increase the regenerative response to injury in aging muscle.

Rationale: L-arginine is a naturally occurring amino acid that is produced by the body and utilized in a variety of physiological functions, ranging from the urea cycle to the production of NO. It is this role as a precursor to NO that warrants the investigation of its possible effects on muscle regeneration. Additionally, L-arginine has been shown to increase muscle regeneration in mice with induced muscle injury, a promising development (25).

Hypothesis 2: L-arginine supplementation will increase the regenerative response to muscle injury in aging subjects.

Strengths and Limitations

The principal strength of this *in vivo* study is the practical clinical significance afforded by doing a whole animal model of muscle injury. This is in direct contrast to an *in vitro* study, where the isolation of cells outside the animal can lead to potentially unrealistic results. In addition, the model chosen enables a more accurate determination

of the inherent clinical efficacy of the substances chosen. This is also the first study to investigate the benefits of L-arginine and ISDN supplementation on aged muscle response to injury.

This research design is limited in its ability to determine the mechanism of potential effects of the dietary treatments on muscle regeneration. Nitric oxide signaling can affect a myriad of events in the intact animal including satellite cell activity, muscle blood flow, protein synthesis, and mitochondrial respiration. Effects of L-arginine and ISDN could result from any of these or other changes. Therefore, future studies will be required to determine the cellular mechanisms underlying our results. Other limitations include the use of the mouse as a model of human physiology and restriction of observations to male mice at 3 and 24 months of age.

Clinical Significance

The loss of skeletal muscle regenerative potential with aging is a serious clinical problem. Age-related declines in overall muscular strength, balance, joint mobility, and visual and hearing acuity lead to increased chances of falls and orthopedic injury. Unfortunately, elderly patients may never fully regain muscular strength following an injury, due to reduced muscle regenerative potential. Therefore, a muscle injury can invoke a cycle of physical decline into frailty characterized by loss of independence and a dramatic reduction in quality of life. The recent recognition that aging skeletal muscle is fully capable of robust regeneration if exposed to the proper environment suggests that nutritional or pharmacological interventions could rescue the regenerative potential of aging skeletal muscle. Based on the known role of nitric oxide in muscle regeneration, and previously published reports of NO donors and L-arginine effects on isolated myofibers from aging mice, we have tested the efficacy of L-arginine and ISDN

supplementation in an in vivo model of muscle injury in aging mice. If effective, these supplements could have immediate impact on the health and wellness of elderly individuals.

CHAPTER 2
LITERATURE REVIEW

Foreword

The recent finding that skeletal muscle in aged individuals can exhibit a near complete regenerative response when exposed to young serum has profound implications on the realm of sarcopenia research (13). This discovery clearly indicates there are some unknown factors in the adolescent serum that are allowing for significantly increased recuperation that are either degraded or simply absent in the aged environment. Therefore, we investigated the potential of supplements such as L-arginine or ISDN to attenuate such decreases in aged animals in hopes of providing a model that could be extrapolated to the problem of sarcopenia in humans.

Overview of Myogenesis

Myogenesis is a very complex and multi-faceted process, from muscle degeneration and inflammation, to satellite cell activation and proliferation. Myogenesis begins with an injury to the muscle, mechanical or otherwise, that damages the myofibers and causes an inflammatory response. This results in activation and proliferation of satellite cells, muscular stem cells whose role and characteristics will be thoroughly discussed later in this review. These cells are then directed by chemotaxis to the site of myotrauma, where they either fuse to the existing fiber and create a hypertrophic response, or fuse with other satellite cells to form new myotubes, a hyperplasic response. In both cases a fraction of the newly differentiated fiber's satellite cells return to quiescence and myogenesis is complete. Therefore, the satellite cell, being responsible for such a large portion of the regenerative process, holds the key to

understanding and attenuating the factors responsible for declining muscle function in the aging population.

Satellite Cell Delineation

Satellite cells are progenitor cells situated between the basal lamina and the myofibers and are derived during embryonic development from the dermomyotome, an epithelial-like derivative of early somites that surround the notochord. Recent work by Gros et al. has reaffirmed the status of satellite cells as exclusively somatic and not hematopoietic in origin (18). It has been long established that satellite cells are legitimate stem cells, meeting the requirements of progeny and self-renewal. These properties are what makes them so valuable, and have generated immense scientific interest in their possible therapeutic applications. In addition, recent research has shown their differentiation potential is not limited merely to skeletal muscle, but can be extended to adipocytes and osteocytes when exposed to the correct growth factors (2). This exciting discovery is not without its flaws, however, as cross-contamination from non-myogenic cells is a concern. Furthermore, satellite cells have been purported to not only be multipotent, but heterogenic as well, even between those cells inhabiting the same myofibers. Satellite cells in any given fiber can differ in their differentiation capacity, specificity, and ability to proliferate and regenerate (26). However, despite our knowledge of their heterogeneity, many questions remain, not the least of which are in regard to age-related changes. It has been demonstrated that satellite cells do indeed lose efficacy in aged populations, likely due to a cascade of systemic effects in the circulation as well as overall decreases in number and function (6). Therefore, elucidating the factors responsible for the deterioration of the regenerative environment

with age and its corresponding role in satellite cell activity and efficacy is a primary focus within the study of myogenesis, and more specifically, muscle regeneration.

The First Stage: Degeneration and Inflammation

The first component of muscle regeneration is degeneration and inflammation of the myofibers. In exercise or other injurious stimuli, muscle fibers are damaged and become necrotic. These insults damage not only the structural proteins of the fiber itself, but the resulting transient increase in sarcolemmal membrane permeability enhances the fibers' susceptibility to proteolysis, including calpain activation. This poration of the plasmalemma also results in secretion of muscle proteins such as creatine kinase and troponin I into the blood, which explains why these molecules are the most commonly used biomarkers of muscle injury, especially in conditions such as muscular dystrophy and myocardial infarction (29)(30). Concomitant to the protein release, inflammatory cells are directed by chemotaxis to the site of muscle injury, with the first responders being neutrophils, increasing in as little as one hour after damage (23). Following the infiltration of the neutrophils, macrophages travel to the necrotic area through the vasculature and reach their peak about 2 days post-injury. Not only do these cells clear the area of compromised sarcolemmal, contractile, and other tissue, *in vitro* research has implicated them in activation and chemoattraction of satellite cells (8). At this point, regeneration of the myofiber can begin and satellite cells play an integral role in the process.

The Second Stage: Regeneration

Muscle regeneration is largely mediated by the activity of satellite cell. Nitric oxide, acting in an enabler role, increases the activity of matrix metalloproteinases (MMP's), which lead to augmented expression of hepatic growth factor (HGF), widely

considered an integral activator of the satellite cell. Upon the first few hours of the ensuing satellite cell activation, muscle regulatory factors (MRF's) MyoD and Myf5 are upregulated, causing satellite cells to progress to the myoblast stage, where rapid proliferation occurs. Myoblasts then withdraw from the cell cycle and express the late MRF's myogenin and MRF4, transforming into terminal myocytes. However, some myoblasts fail to differentiate and instead reprise their role as quiescent satellite cells, thought to serve the purpose of self-renewal. The differentiated myocytes then merge with one another to form "multinucleated syncytium, which eventually mature into contracting muscle fibers (9)." This marks the completion of myogenesis, but this to say nothing of the balance between regeneration and fibrosis.

Hanging in the Balance: Regeneration and Fibrosis

The degree of muscle healing has been defined as the balance between regeneration and fibrosis. The main fibrotic unit is the fibroblast, accompanied by collagen types I and III, as well as glycoproteins and proteoglycans. The fibroblast has the ability to indefinitely promote the inflammatory response, and despite the aforementioned mitogenic characteristics of macrophages, excessive amounts of certain secreted factors such as transforming growth factor-beta-1 (TGF-β1) inhibit satellite cell differentiation and as a result attenuate myogenesis as a whole (7). TGF-β1 has been shown to be culpable in the in scar formation during myogenesis, stimulating the synthesis of extracellular matrix proteins while concurrently blocking their proteolysis. Compounding these deleterious effects is its promotion of satellite cell differentiation into myofibroblasts that produce additional collagen I. In addition, TGF-β1 has also been implicated in diseases involving fibrosis such as muscular dystrophy and inflammatory myopathy; its presence has been localized to those areas between

myofibers as well as regions of neutrophil and macrophage infiltration. Furthermore, Smith et al. determined that TGF-β protein precursors were elevated 2 days after muscle injury (28). This is consistent with the peak activity of macrophages, suggesting a correlation between macrophage concentration and TGF-β expression. This could be of special interest to those studying fibrotic muscle disorders, and has potential for therapeutic applications. It has also recently been postulated that nitric oxide and TGF-β1 have opposing effects. In a study investigating crush-injured muscle and synovium of controls versus those of rats treated with n-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, TGF-β levels in the regenerating tendons of the control animals were elevated at first but subsided after three weeks. In stark contrast, the TGF-β levels of their L-NAME treated counterparts were interminably elevated, with no signs of recession even after five weeks. This relatively perpetual elevation of TGF-β in recovering muscle has been well documented to be correlated with increased collagen secretion by fibroblasts, the accretion of which results in the aforesaid deficits in muscle regeneration and tendon healing (15). Additionally, it has been shown that subjects provided with L-NAME two hours after injury have increased expression of TGF-β a week later, furthering the assertion of an NO-inhibitory mechanism (17). Along with the aforementioned NO-dependent upregulation of MMP's, it has been shown that the MMP's play a crucial role in degrading many of the products of increased TGF-β expression, such as the collagens and proteoglycans (33). It is well within the realm of possibility that these MMP's are the "enforcers" of the extracellular matrix, policing its makeup and preserving its integrity, in direct opposition to the "criminals" begotten by TGF-β. Therefore, the balance between regeneration and fibrosis, and, to a larger

extent, muscle recovery as a whole, is largely regulated through the NO/TGF-β ratio. In other words, it is the incessant battle for supremacy between satellite cell activation and collagen deposition that ultimately determines the fate of the muscle.

Getting to the Source of Age-Related Deficits

Recent research has brought Notch/Delta signaling to the forefront of the conversation when it comes to regulation of satellite cell activation. Notch has been identified as a regulator of stem cell differentiation during embryonic development, and has a significant role in muscle regeneration. Notch signaling is initiated when one of its ligands, such as Delta, interacts with the transmembrane Notch receptor. This results in a cascade of reactions and cleavages that culminates in the transcription of myogenic proteins via the Hey and Hes genes (Figure 1)(1)(31).

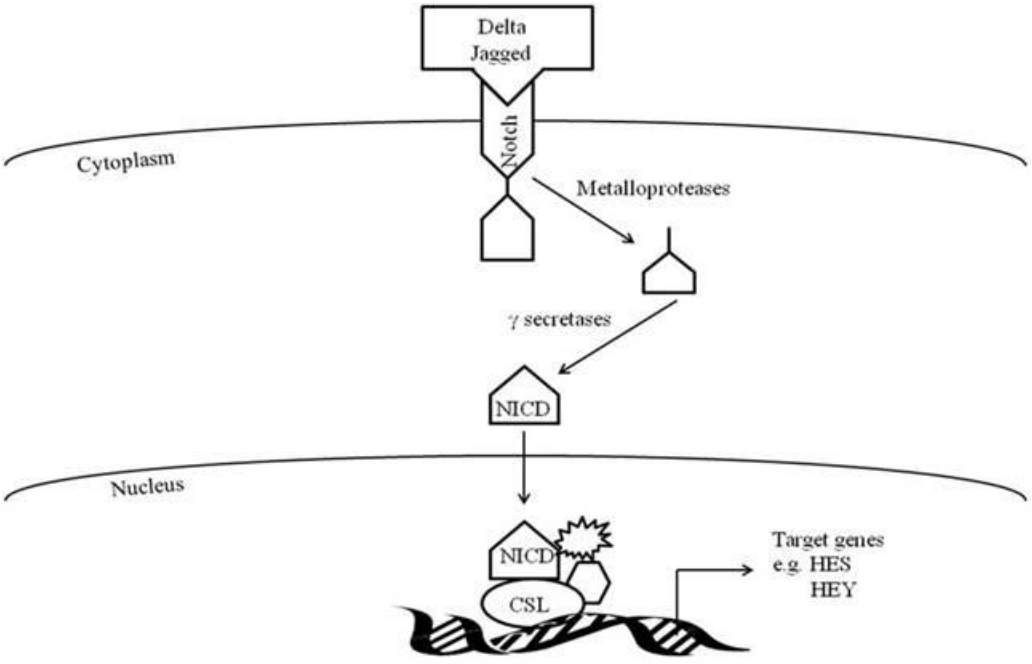


Figure 2-1. Overview of Notch/Delta signaling

Not only has it has been shown that Notch signaling components are upregulated in the hours to days following muscle damage, their increased activity has been

localized to the satellite cells and adjacent muscle fibers themselves. This knowledge served as the impetus for further elucidation of their role, and it is now known that augmented Notch signaling promotes satellite cell proliferation while simultaneously arresting myoblast differentiation. However, when Notch signaling is inhibited by its antagonist, Numb, proliferation is suppressed and differentiation increased. Moreover, Conboy et al. demonstrated that satellite cells in aged muscle failed to upregulate Delta expression in response to injury, significantly hampering regeneration, and more specifically, the proliferation of satellite cells. It is notable that increased Delta expression was correlated with decreased Numb expression. The investigators then administered Jagged-Fc fusion protein, a specific inhibitor of Notch activity, to the young animals and similar detriments in muscle regeneration to that of the old tissue were observed. Furthermore, they specifically activated Notch signaling using an antibody and it elicited an almost completely identical regenerative response between the young and old muscle (11). In a separate experiment, Conboy et al. used a heterochronic parabiosis model to determine if exposure to a young systemic environment could rescue the regenerative capacity of old muscle. Strikingly, they demonstrated that, when exposed to young serum, aged muscle regenerated in a similar, robust fashion to that of its younger parabiont, likely due to the measured increase in Delta expression. In contrast, the old isochronic parabionts were characterized by lackluster muscle regeneration and satellite cell activation, with notable fibrosis, a response typical of old muscle. Likewise, the young heterochronic parabionts exhibited slightly decreased upregulation of Delta, suggesting the old serum has a negative impact on the Notch pathway. In conjunction with these measurements, hepatocyte proliferation was also

recorded, and the results were similar: increased progenitor cell proliferation in old heterochronic parabionts in relation to their old isochronic counterparts (12). Therefore, it can be postulated that the observed age-related difference in Notch activation, specifically by the ligand Delta, is the rate-limiting factor present in aged serum and is responsible for its inability to activate satellite cells, thus limiting regenerative potential in aged animals relative to their younger counterparts. Clearly, more research needs to be done to fully elucidate the mechanisms, but the therapeutic potential for the rescue of progenitor cell function is unbounded.

Osteopontin (OPN) is an extracellular structural protein, originally discovered in bone, which has a wide variety of functions including regulation of inflammation and wound healing. It can also function as a pleotropic cytokine, expressed primarily in macrophages, that has profound effects on muscle regeneration as well as a myriad of other processes (21). Furthermore, Paliwal et al demonstrated that when OPN was neutralized, myogenic response increased. Also of note was their finding that, while the quantities present in each respective muscle were equivalent, young CD11b+ macrophages exhibited a rescue effect upon muscle regeneration compared to the old macrophages when all other factors were controlled for (27). This discovery is very intriguing especially when the Notch experiments are taken into account. It is reasonable to postulate that OPN could be one of many significant contributors to reduced Notch-Delta signaling, and thus reduced regeneration, in senescent muscle.

L-Arginine and ISDN

Exogenous NO-donor supplementation is thought to increase the efficacy of the muscular regenerative process as NO plays a role in multiple stages of recovery. L-arginine is the primary substrate for the synthesis of NO, acting through the nitric oxide

synthase enzyme (NOS) to create NO and citrulline, which than can be converted to L-arginine and begin the process anew. A recent study has shown that L-arginine supplementation attenuates the decline in NO content of hind-limb suspended rats, as well as ameliorates the atrophic response relative to their control counterparts (20). Furthermore, an investigation by Neto et al. demonstrated that L-arginine enhances muscle regeneration in an envenomation model, with treated animals showing a greater response than untreated controls but not recovering to the level of the uninjured cohort (25). The precise mechanism for these effects remains unclear, but it seems to be related to NO production by way of NOS. In addition to upregulating NO production, L-arginine supplementation has also been shown to be angiogenic. Therefore, it is not unreasonable to theorize at least some of its positive effects on muscle regeneration stem from its capacity to restore the damaged vasculature surrounding the muscle. Nevertheless, a major obstacle inhibiting L-arginine from achieving high efficacy is its propensity to cause undesirable gastrointestinal side effects, such as nausea and diarrhea, especially in higher doses (16).

Another NO donor, Isosorbide Dinitrate (ISDN), has recently entered the supplement conversation largely due to its status as a direct NO donor, rendering NOS activity irrelevant. In contrast with L-arginine, ISDN has very few side effects, making it an attractive alternative. In a study by Marques et al., *mdx* mice tibialis anterior muscle was injected with bupivacaine and subsequently supplemented with ISDN, with an additional group administered a purely vasodilatory agent. The group given the ISDN showed a 20% increase in regeneration over both of the other groups, validating the assertion that the increase in NO is responsible for the augmented regeneration and not

the vasodilatory mechanism (22). Both of these NO-donors have established vasodilatory effects on the vasculature, and are becoming increasingly eminent in the field of muscle regeneration. However, future research focused on the actual mechanism and true efficacy of the augmented regeneration is needed to clear the ambiguity surrounding these two molecules.

Conclusion

To say the process of myogenesis is mediated by a myriad of factors is an egregious understatement. From macrophages, to satellite cells, and even the recently prominent Notch signaling, it is clear we have significant work ahead of us in truly understanding the process of muscle regeneration. Nevertheless, recent research has provided the impetus for further investigation into this convoluted pathway, and its elucidation will surely prove invaluable. Furthermore, new supplementation options to improve muscle regeneration could have widespread clinical influence in the future, and the potential to rescue aged muscle with young serum is beyond promising. All of this progress makes it irrefutably evident that the means to attenuate the decline in skeletal muscle as we age exist; we just have to find them.

CHAPTER 3
METHODS

Experimental Design

The University of Florida Institutional Animal Care and Use Committee approved the protocol of this study. Mice were exposed to a control diet, or a diet supplemented with either L-arginine or isosorbide dinitrate (ISDN) for one week prior to injury to the right tibialis anterior by injection of cardiotoxin. The left tibialis anterior served as a contralateral non-injured control. Mice were monitored daily for food intake and changes in body weight. At 5 and 10 days post-injury, mice were killed and the tibialis anterior muscles removed for subsequent histological sectioning. The design is illustrated in Table 3-1.

Table 3-1. Experimental Design

	5 –day Recovery	10-day Recovery
Young Adult Control	*n=4*	*n=4*
Old Adult Control	*n=4*	*n=4*
L-arginine	*n=4*	*n=4*
ISDN	*n=4*	*n=4*

Animals

Male C57 mice were purchased from the NIA aged rodent colony at 3 and 23 months of age. The animals were housed in the SPF facility in the J. Hillis Miller Health Science Center at the University of Florida and maintained on a 12:12h light-dark photoperiod. Mice were housed for at least one week prior to experiments.

Experimental Diets

To begin the protocol, mice were randomly assigned to isocaloric low-protein (10% kcal from casein) diets supplemented with either 1) L-arginine (2% kcal from L-arginine), 2) a mixture of amino acids (2% kcal) specifically design to exclude L-arginine and other amino acids involved in endogenous production of L-arginine (Control diet), or 3) the control diet supplemented with isosorbide dinitrate (ISDN, 4 g/kg diet). Mice began experimental diets 7d prior to myotoxin injections, and continued on these diets throughout the recovery period.

Experimental Protocols

Cardiotoxin Injury

Muscle regeneration was induced by a direct transcutaneous intramuscular injection of cardiotoxin from *Naja mossambica mossambica* venom (Sigma Chemcial) in physiological saline, into the tibialis anterior (TA) muscles of adult mice. Injections were performed unilaterally, with the contralateral muscles serving as non-regenerating controls. Mice were anesthetized with 5% inhaled isoflurane in oxygen, and the lower legs cleaned with 70% ethanol. A 28-gauge needle was then inserted through the skin and into the tibialis anterior muscle along the longitudinal axis of the muscle to inject ~20µL of sterile saline containing 10 µM cardiotoxin. The needle was withdrawn and re-inserted into the muscle multiple times at slightly different angles, injecting ~20µL each time until a total of 100µL of cardiotoxin solution had been injected. Contralateral legs were prepared identically to the injured leg, but without toxin injection.

Tissue Removal and Preparation

At the end of the protocol, the tibialis anterior muscles from each leg were removed under general anesthesia (5% isoflurane), weighed, embedded in OCT

(Tissue-Tek) compound at resting length, and frozen in liquid nitrogen-cooled isopentane. Samples were then cryosectioned at 10 µm for histological analyses. Following cryosectioning, muscle sections were affixed to glass microscope slides. Multiple serial sections were collected on separate slides for independent staining procedures, as described below.

Hematoxylin & Eosin Stain

Muscle sections were stained in accordance with standard hematoxylin and eosin protocols. Briefly, slides with muscle sections were air dried at room temperature for 30 min before incubation in Harris Hematoxylin solution for 5 min. After rinsing in running tap water, slides were immersed 6 times in acid alcohol (1% HCl in 70% ethanol), washed again in running tap water, and immersed briefly in ammonia water (1mL NH4OH in 1 liter of dH2O). This was followed by another rinse in water and incubation in Eosin Y solution (5% aqueous solution) for 3 min. Slides were then rinsed again in running water, dehydrated by immersion in progressively increasing concentrations of ethanol (35, 50, 75, and 100% EtOH), and cleared by repeated immersion in CitriSolv (Fisher Scientific). Finally, dried muscle sections were covered with permount and cover-slipped.

Analyses

Image Analyses

Microphotometric digital images of stained muscle sections were captured using a Zeiss Axiovert200 light microscope (Thornwood, NY) and Qimaging RETIGA EXi digital camera (Surry, BC, Canada) and software (IPLab3.6.5, Scanalytics, Rockville, MD). H&E stained images were evaluated for myofiber dimensions (cross-sectional area and nuclei/fiber) and myofiber density (myofiber area/total area) using ImageJ

imaging software (NIH). Two images of each muscle section were captured. Within each image all fibers completely contained in the field of view were analyzed for area and nuclear content. Regeneration Index (RI) was tabulated through quantitative analysis of centrally situated nuclei divided by total number of nuclei. Further, the total number of nuclei per muscle cross-sectional area was determined by counting all nuclei in the field of view. Area measurements were calibrated using the captured image of a stage micrometer at the same magnification (40x) used for muscle section analysis.

Statistical Analyses

Myofiber cross-sectional area, nuclei per fiber, regeneration index, and nuclear density per muscle area from each analyzed image was averaged to yield one observation per variable per muscle section. These data were then analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Significance was established at $P<0.05$.

CHAPTER 4
RESULTS

NO-Augmenting Supplements as a Method of Nullifying Age-Associated Muscle Wasting

In this study, we investigated the potential of two NO-augmenting supplements, L-arginine and isosorbide dinitrate (ISDN) to combat the oft-observed deficit in myogenic capacity of elderly individuals. Our two main experimental groups, five- and ten-days post CTX injection, provided two very dichotomous snapshots into the chronology of the regenerative process. Our four subgroups, young control (YC), old control (OC), old arginine (OA), and old ISDN (OI), allowed us not only to compare the supplemental groups with the old controls, but with the young controls as well to provide an index of restored myogenic capacity. Subject and sample metrics are shown in Table 4-1 and Table 4-2. Representative H&E images of all groups are shown in Figure 4-1.

Table 4-1. Average tibialis anterior (TA) and body masses (BM) for all groups. Values are mean±SEM.

	5 Day			10 Day		
	CTX TA(mg)	CL TA(mg)	BM (g)	CTX TA (mg)	CL TA (mg)	BM (g)
YC	43.8±4.3	63.5±2.8	30.3±1.0	46.0±4.3	51.7±2.6	29.4±0.2
OC	46.5±1.9	60.5±1.5	37.4±1.5	39.9±3.0	47.9±0.8	35.6±0.2
OA	45.6±3.8	62.0±3.5	35.9±1.4	41.8±2.0	55.0±4.7	36.2±2.0
OI	46.8±1.4	60.8±2.5	36.0±0.9	45.1±6.3	57.1±1.6	32.2±2.1

CTX=Cardiotoxin, CL=Contralateral non-injured

L-Arginine Supplementation Increases the Regenerative Response of Senescent Muscle at Five Days Post Injury

Our five day data illustrate that L-arginine supplementation had a significant effect on muscle regeneration. The Regeneration Index (RI), calculated as centrally located nuclei per total nuclei, provides a gross but nonetheless powerful assessment of the balance between cell proliferation (including both myogenic and infiltrating immune

cells) and myogenic cell fusion. The RI of the OA group was significantly different from that of the OC group, and was nearly equal to that of the YC group (Figure 4-3A). However, the average cross sectional area and the total fiber area showed no change (Figure 4-2A; Figure 4-4A). In addition, the response at ten days was equally insignificant across all categories (Figure 4-2B; Figure 4-3B; Figure 4-4B). Also of note, the average cross sectional area of the cells in the OA group was, in general, less than those of the other groups. This is due to the fact that, on average, the OA samples were characterized by more abundant, but smaller cells, than those of the other groups, as noted during observation and data collection.

Table 4-2. Recorded and calculated parameters of regeneration for all samples.

	TACXA	Fibers	Nuclei	RI	Central Nuclei	Total Area
			Five days post CTX injection			
YC	963±77	42.3±2.4	490±25	0.194±0.007	95.1±4.3	40134±3382
OC	624±46	52.8±7.0	608±11	0.143±0.162	87.1±11.4	32100±3382
OA	638±54	52.6±4.1	586±22	0.198±0.004	116.0±6.1	32919±456
OI	668±63	40.5±3.2	531±13	0.178±0.015	94.4±6.5	28262±4412
			Ten days post CTX injection			
YC	1997±258	49.8±6.5	481±30	0.289±0.013	128±11.1	94417±4337
OC	1289±164	66.4±8.4	465±47	0.263±0.014	119±8.5	84843±12029
OA	978±107	93.8±10.2	549±47	0.250±0.026	135±11.3	89406±7658
OI	1314±124	82.6±7.8	574±41	0.243±0.017	148±11.9	103229±4558

Values are mean±SEM. CXA=Cross sectional area, RI= Regeneration Index

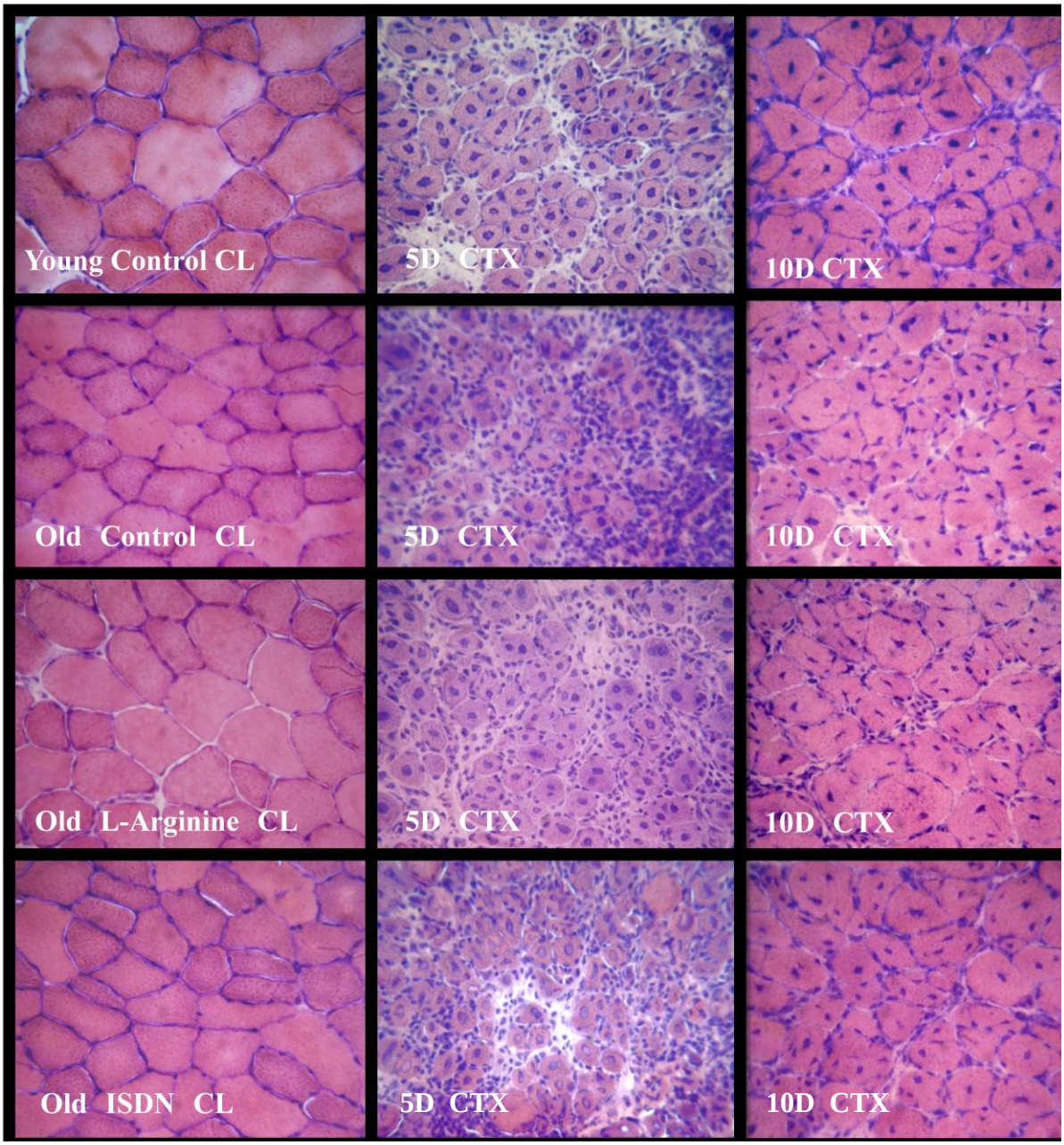
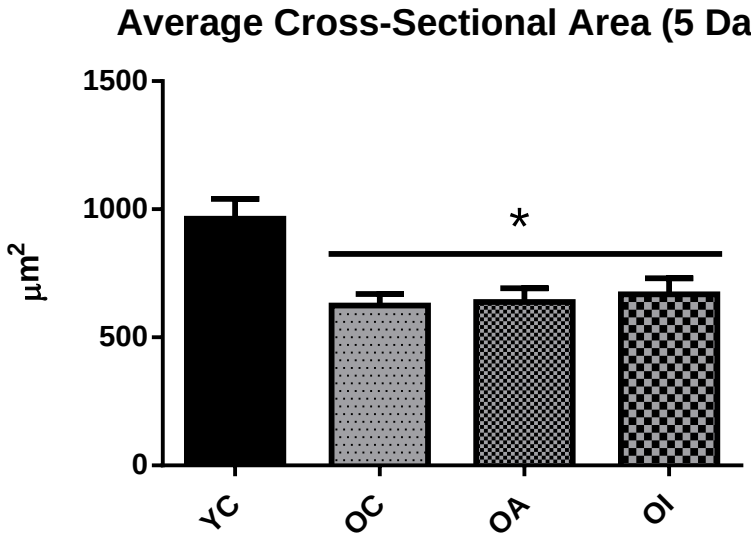
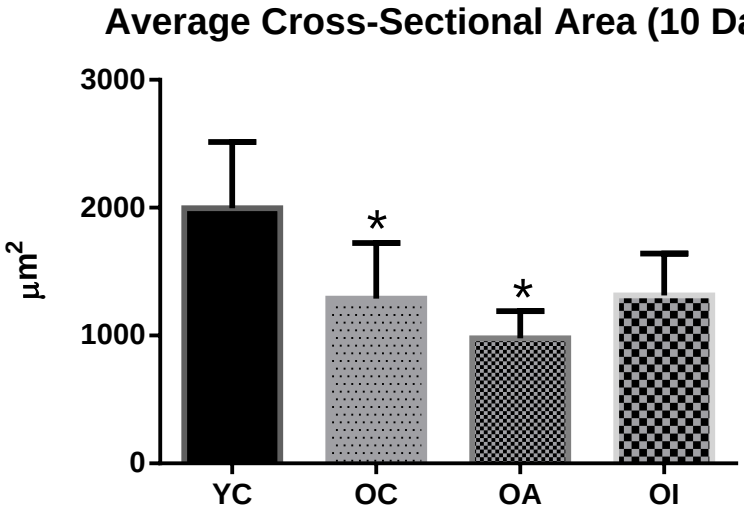


Figure 4-1. Representative H&E sections from all groups. TA muscle was removed at 5 or 10 days and analyzed using ImageJ.

Figure 4-2. Average TA cross sectional area post CTX injection. *Significantly different from YC ($p<0.05$). A) Five days post CTX injection. B) Ten days post CTX injection.

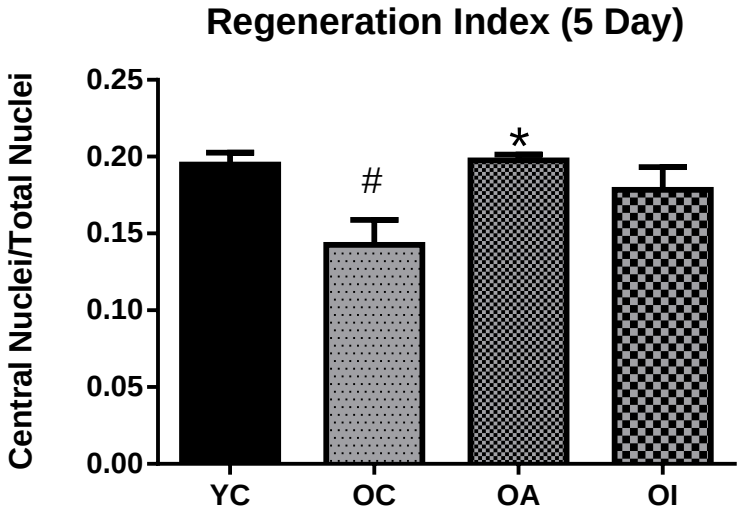


A

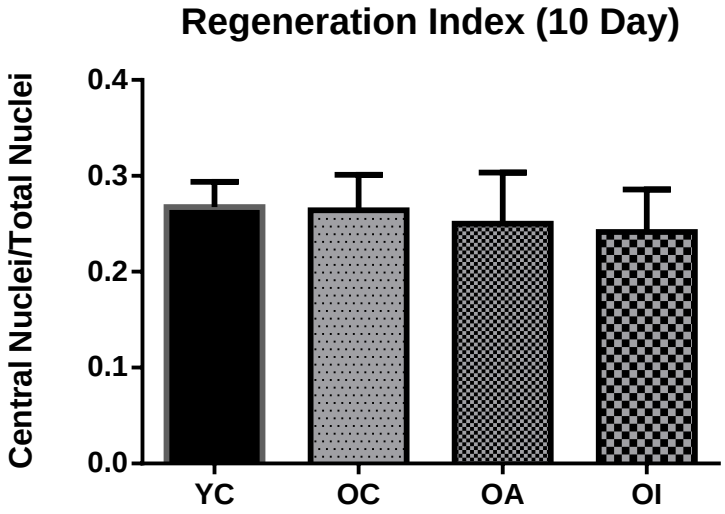


B

Figure 4-3. Regeneration Index (RI) of all groups post CTX injection. [#]Significantly different from YC ($p<0.05$). *Significantly different from OC ($p<0.05$). A) Five days post CTX injection. B) Ten days post CTX injection.

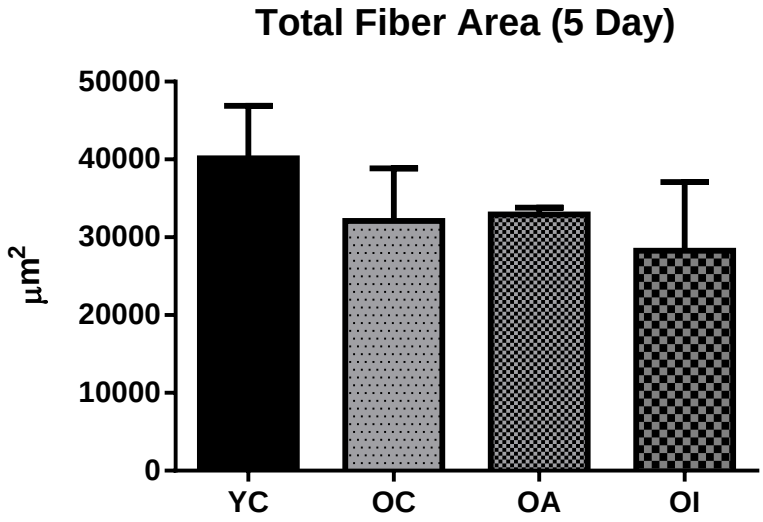


A



B

Figure 4-4. Total fiber area for all groups post CTX injection. A) Five days post CTX injection. B) Ten days post CTX injection.



A

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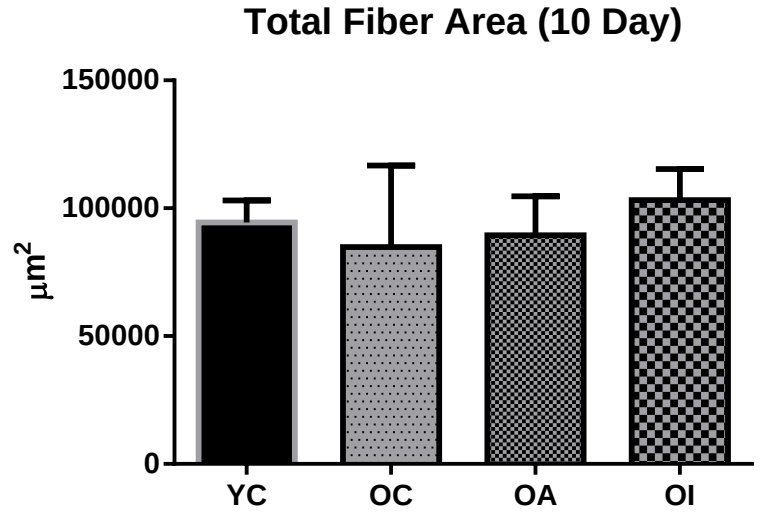

B

ISDN Supplementation Shows a Positive Trend Toward Increasing the Myogenic Capacity of Senescent Muscle

Treatment with ISDN showed a positive, yet not statistically significant response on the RI of the subjects at five days post CTX injection (Figure 4-3A). However, similar to the arginine treatment group, the ISDN supplementation at ten days seemed to have

negligible effects across the board (Figure 4-2B; Figure 4-3B; Figure 4-4B). ISDN also seemed to have a slightly more positive effect than L-arginine on TA cross sectional area at five days even though the total fiber area was much lower, possibly due to the relatively large disparity in fiber number (Figure 4-4A; Table 4-2).

CHAPTER 5
DISCUSSION

Principal Findings

The impetus for this study was provided by prior forays into the investigation of *in vitro* L-arginine and ISDN supplementation, and the resulting increase in NO levels, as a means of combating the oft-reported phenotypic inability of aging muscle to regenerate in any sort of accordance with that of its younger counterparts. In 2008, Betters et al. reported an increase in satellite cell activation as a result of L-arginine supplementation using a cell culture model, as well as a full rescue response when HGF was administered. However, the results obtained with L-arginine failed to demonstrate a recovery to youthful levels, but reported a nonetheless important partial vitiation of the deficit. They then postulated that the difference in the regenerative capacity of aging muscle fibers was largely a construct of the decreased sensitivity of the older satellite cells to NO, and not the inherent quantitative presence of NO itself, although the augmented presence can have limited positive effects (4). This work, in addition to that conducted by Marques et al. demonstrating the potential for ISDN to improve myogenesis in an mdx model, largely provided the foundation for the current study to investigate the ability of NO-donor supplementation to at least partially mitigate the aforementioned myogenic deficit. This study is the first to demonstrate the potential for exogenous L-arginine and ISDN supplementation to improve senescent muscle regeneration at five days post-muscle injury. Our results, despite some of them lacking proper statistical significance, display a strong trend toward the chosen supplements facilitating muscle regeneration. As such, these results tentatively confirm our prior hypotheses.

Matrix Metalloproteinases are Key Modulators of Satellite Cell Function

The deficit in aging satellite cell function has been well-documented, and recent evidence has implicated the aging environment, acting to retard the activation of the satellite cell, as the culprit, rather than the satellite cell itself. Rando and colleagues have illustrated on multiple occasions that this is the case, often using young serum to rescue the regenerative potential of the aged muscle and produce levels of satellite cell activation akin to the levels achieved in youth (12). In addition, as noted by Yamada et. al, a family of ubiquitous endopeptidases known as matrix metalloproteinases (MMPs), especially MMP-2, have the ability to cleave hepatic growth factor (HGF) from its extracellular bind, freeing it to activate satellite cells (33). Moreover, it has been shown by multiple studies that MMP dysregulation is a common problem associated with age and is involved in the formation of many serious diseases, such as cancer and cardiovascular disease (3)(10)(19)(32). Combined with the knowledge that senescent satellite cell activation is completely rescued through HGF supplementation and the fact that proper MMP activation is NO-dependent, it would follow that the primary mechanism through which regeneration is increased in elderly subjects could very well be the corresponding rise in proper activity of the MMPs achieved through NO supplementation and the resulting increases in bioavailable HGF. Therefore, the data suggests the relatively robust regenerative capacities exhibited by the treatment groups in our data at five days are at least partially a result of improved MMP signaling through facilitated NO production.

NO Supplementaion as a Means of Sarcopenia Treatment

Despite the rampant prevalence of sarcopenia in modern society, there has been a relative lack of research into possible treatments and therapeutic methods. A few

studies, however, have been promising using mdx models of sarcopenia. This clinical model may be considered a form of accelerated aging since mdx mice lack sufficient levels of nNOS, the principal source of muscular NO, and exhibit severe deficits in muscle regenerative potential at a relatively young age. A stage I clinical trial conducted by D'Angelo et al. demonstrated the possibility that a combination drug of ISDN and ibuprofen may be effective in treating sarcopenia. Many of the patients included in the trial not only had improved motor function, but also had decreased serum levels of TGF-β (14). As mentioned earlier, increased TGF-β levels are associated with decreased satellite cell activation and an opposing increase in collagen deposition and fibrosis in the muscle, common characteristics of senescent myofibers. It is therefore not unreasonable to hypothesize that the positive results of ISDN in this study could be due to the increased levels of NO resulting in enhanced MMP activation and signaling. Combined with our data, this suggests that supplementation with ISDN could be an effective method in the prevention and attenuation of the muscle wasting that occurs in sarcopenia. Furthermore, a study by Tidball et al. has shown that magnified NO production effectively inactivates the proteolytic actions of calpain, thought to be a prominent player in sarcopenia. They postulated that through the increased activity of nNOS, calpains were S-nitrosylated, significantly attenuating the loss of muscle tissue usually associated with aging. Taken collectively, these studies along with our data indicate that both ISDN and L-arginine supplements could be potentially efficacious therapeutic interventions in the treatment of sarcopenia.

Possible Side Effects of L-Arginine

Despite its clear budding promise as a solution to the rampant issue of sarcopenia, it would be a critical omission to not discuss the potential side effects of L-

arginine supplementation. As previously mentioned, common side effects reported by those administered L-arginine include nausea and diarrhea, as well as numerous other symptoms associated with gastrointestinal distress (16). However, there has been a relatively recent revelation that prolonged L-arginine supplementation results in increased fibrosis. Tidball et al. recently conducted a study investigating the effects of long-term L-arginine supplementation in mdx subjects on muscle fibrosis, and the results were striking: fibrosis increased markedly both in the skeletal muscles as well as the myocardium. This occurred as a result of the upregulation of arginase, an enzyme prevalent in the metabolism of arginine, and its effect on macrophages, causing fibrosis of the muscle tissue (32). It is important to note, however, the facts that mdx subjects are by nature characterized by a dearth of intrinsic NOS and that NOS is a primary competitor of arginase. Therefore, caution should be demonstrated before extrapolating these results to healthy individuals, because the lack of dystrophin at the membrane could very well be a confounding factor, and much more research needs to be done in this area to assure correct application. Overall, the future of L-arginine supplementation as a potential option in the treatment of senescence-related muscle wasting is very bright, but one that is wrought with questions and we suspect many more years of inquiries into its far-reaching effects will be required to elucidate its full potential.

Future Directions

When the current literature is taken collectively into account with our data, it is clear that there is much work to be done. First, any future studies in our lab concerning this matter would need to include contractile data to ascertain the effects NO-donor supplementation has, if any, on functional recovery. Second, many more time points are needed to address the different stages of muscle recovery and the potential impacts

supplements are having at the given points. Our results show a clear effect at five days, but the effect is nearly nonexistent at ten days. Repeated studies in this area would help to solidify the knowledge base on this matter, and paint a clearer picture of the cellular events between those two time points. Further, as with any animal model, extrapolating these results to humans is wrought with hazards and human research would be the final step in verifying the efficacy of such supplements in humans.

Conclusions

Based on our data and the body of work currently presented in the literature, we believe that ISDN and L-arginine supplementation as a means of attenuating the effects of sarcopenia definitively merits further investigation. While not without its seemingly infinite facets, the culprits responsible for the myogenic deficit in the elderly are slowly coming into view. We postulate that nNOS and MMPs are dysregulated in the elderly, account for a significant portion of the regenerative dearth observed in sarcopenia, and that L-arginine and ISDN supplementation have the potential to partially mitigate that deficit through the promotion of nNOS presence and proper MMP function.

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BIOGRAPHICAL SKETCH

Kevin James Miller was born in 1988 in Dunedin, Florida to Joseph and Patricia Miller. He attended and graduated from Olympia High School in 2007 and begun attending the University of Florida the following August. He graduated with a bachelor's of science in exercise physiology in 2011, and decided to return that fall for his master's in the same subject, under the supervision of Dr. David Criswell. He has also worked for UF recreational sports for 5 of his 6 years at the university, and has been a certified personal trainer since 2011. He was recognized as the 2011-2012 UF personal trainer of the year in April 2012, and was afforded the opportunity to present a lecture at the 2013 Evolve fitness symposium. Following graduation, he will be employed at the Equinox fitness club in Coral Gables, Florida, as a personal trainer, pursuing his dream of helping any and everyone fully realize and redefine their physical potentials.